

Influence of the stretching temperature on the VOC profile and odor intensity of high moisture mozzarella: a model study

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Abstract

The impact of the stretching phase on the volatile compounds and odor intensity of high moisture mozzarella was investigated. Three trials were done using milk with different microbiological quality for the preparation of control and aseptic cheeses at two different stretching temperature. The SPME-GC/MS analysis showed that stretching caused a decrease of compounds from curd to cheese in the aseptic samples due to volatilization or solubilisation into the stretchwater. It was not observed in the control mozzarella due to the formation of microbial-derived compounds during the cooling phase. As to the effect of the temperature, the samples stretched at 70°C had the highest amount of VOC, except for those made from high quality milk. The formation of new volatile compounds due to stretching remained doubtful. The control samples stretched at 70°C had the highest odor intensity, whereas stretching at 90°C resulted in a more intense odor in the aseptic cheeses.

Keywords: High moisture mozzarella, Stretching temperature, Volatile organic compounds, Sensory evaluation, Paired comparison.

1. Introduction

The stretching phase is a fundamental step in mozzarella manufacturing. During this operation, the curd is soaked into hot water and thoroughly kneaded by hand or machine, with consequent deep physical and chemical changes. In fact, both heating and kneading promote the conversion of the para-casein network into a melted, fibrous matrix in which calcium creates bonds among casein fibers, conferring them the typical string texture. Once this texture is obtained, some void channels appear in the cheese matrix, in which fat and free serum are stuck (Kindstedt, 2007; Kindstedt, Carić, & Milanović, 2004; Lucey & Fox, 1993; Lucey, & Ichihashi, 2009; Mizuno, Matsuda, McMahon & Oberg, 2011; Vogt et al., 2015). For high moisture mozzarella (> 52% moisture content, USDA 2012), the temperature of the water used for stretching can vary in the range 70-90°C, and that of the resulting cheese from 55 to 75 °C, depending on the amount of water used and the time needed to perform the process. The temperature level applied exerts an influence on the features of the finished product, such as the microbiological profile, functionality, and proteolysis (Correia Gonçalves & Cardarelli, 2021). The viability and activity of microorganisms as affected by the stretching temperature have been deeply investigated, with particular focus on pathogens and spoilage bacteria (Buazzi, Johnson, & Marth, 1992; d'Aoust et al., 1988; Kim, Schmidt, Phebus, & Jeon, 1998; Spano et al., 2003). Less information is available about the effect on the chemical-physical characteristics of the cheese, and the available studies only focus on the low-moisture type (< 52% moisture content, USDA 2012), mostly used for dressing pizza. In this type of mozzarella, Ma, James, Zhang, & Emanuelsson-Patterson (2013) found that the higher was the stretching temperature, the higher resulted the protein and fat content, and the lower the moisture. In addition, stretching at low temperature allowed calf rennet and microorganisms to remain active, causing faster protein matrix degradation and softening of the body. Similarly, Kindstedt (2007) and Smith, Carr, Golding, & Reid (2018) reported that stretching the curd at a temperature above 60°C resulted in reduction of residual activity of calf rennet in the cheese, in an inverse proportion to the temperature applied. Gonçalves & Cardarelli (2019) characterized the composition of the stretching water resulting from mozzarella manufactured at 75 and 85°C, and found no differences in pH, calcium, total solids, fat, casein, and salt content. The same was for the chemical composition of the cheese, but proteolysis during storage was differently influenced. Mulvaney, Barbano, Yun (1997) investigated the effect on the cheese texture and found that elasticity decreased as the stretching temperature increased from 57 to 75°C. Rowney, Roupas, Hickey, & Everett (2003) reported that

the application of high temperature increased the diameter of the fat globules stuck inside the protein matrix, which also tended to melt more rapidly and to cause increased amount of free oil. In contrast, Gonçalves & Cardarelli (2020) did not find any differences in the microstructure, texture and thermophysical parameters of mozzarella samples stretched at 75 and 85°C.

From the above literature, it appears that information about the effect of the stretching temperature on the sensory characteristics of mozzarella are lacking. In this perspective, investigating the volatile organic compounds (VOC) might be very useful, since their contribution to the cheese aroma is widely recognized (Curioni & Bosset, 2002). This type of investigation is very challenging, since the VOC formation is deeply influenced by the activity of the curd microbiota, which in high moisture mozzarella mostly takes place during the pre-stretching phases (Natrella, Gambacorta, De Palo, Maggiolino, & Faccia, 2020a). Consequently, it is very difficult to discriminate the volatile compounds eventually formed throughout heat-promoted chemical reactions (i.e. Maillard and Strecker reactions, oxidations, etc.) from those connected to microbial activity. In the present study focusing on high moisture mozzarella, an experimental design was developed to overcome this problem, based on the use of milks with different microbiological characteristics and on the preparation of aseptic samples. By such an approach, the influence of the stretching temperature on the VOC formation and on the odor intensity of the cheese, both in the presence or absence of microbial activity, was investigated.

2. Material and methods

2.1. Experimental design.

The experimental design applied is shown in Fig.1. It involved the preparation of both standard and aseptic high-moisture mozzarella samples from milks of different microbiological quality. To this aim, three batches of bulk raw milk (about 10 litres each) were taken from three different dairy farms located in the province of Bari (Apulia, Southern Italy) and stored for 4 h (“maturation phase”) under different conditions. One batch was kept refrigerated ($4 \pm 2^\circ\text{C}$), the second one was kept refrigerated for 2 h, then was held at room temperature (about 22°C) for the remaining 2 h, and the third batch was kept at room temperature all the time. After assessment of the Total Bacterial Count (TBC) on Bactoscan 8000 instrument (Foss Electric, Hillerød, Denmark), each batch of milk was split into two parts that were transferred into small pots for preparing the

78 mozzarella samples at the laboratory level. Of these two parts, one was processed as it was for preparing a
79 standard cheese (C= control), the other one was processed after addition of sodium azide (0.5:1000/w:w) to
80 obtain aseptic cheese (SA=aseptic). Cheesemaking was done by the direct acidification method, a widely used
81 technique at industrial level, in order to minimize the contribution of microflora to the VOC formation (Natrella
82 et al., 2020a; 2020b). In short, milk was acidified to pH 5.70 with citric acid, then it was heated up to 38°C
83 and added with 200 µL/L calf rennet (153 I.M.C.U, 92% chymosin, Sacco srl, Cadorago, Italy) for coagulation;
84 the coagulum was then cut and the grains were left to aggregate on the bottom of the vat to form the curd. At
85 this point, both C and SA curds were further divided into 2 parts: one was stretched with water at 70°C, the
86 other one with water at 90 °C. The stretching process was standardized by fixing the water to curd ratio at 2:1.
87 After this phase, the cheeses (round shaped, weighing about 50 g) were cooled in chilled water and when the
88 temperature at the core reached 12°C, they were immediately analyzed. At the end of the process, 4 different
89 types of mozzarella were obtained from each type of milk, coded as MozzC70 and MozzC90 (control samples
90 stretched with water at 70 and 90°C, respectively), and MozzSA70 and MozzSA90 (aseptic samples stretched
91 with water at 70 and 90°C, respectively). All cheesemaking trials were done in duplicate.

92 2.2. Analyses.

93 The following chemical analyses were performed: for milk, pH (pHmeter with glass electrode, Hamilton,
94 Reno, USA), gross composition (fat, protein and lactose by Milko Scan FT1, Foss, Hillerød, Denmark) and
95 VOC (Solid Phase Micro Extraction Gas Chromatography-Mass Spectrometry, SPME-GC-MS, as described
96 below); for curd, pH (pHmeter with Polyplast penetration electrode, Hamilton, Reno, USA) and VOC (SPME-
97 GC-MS); for mozzarella, moisture (IDF, 1986), total protein (Kjeldhal method), fat (Gerber method), VOC
98 (SPME-GC-MS) and sensory analysis (as described below).

99

100 2.2.1. Volatile organic compounds analysis.

101 VOC were extracted by solid-phase microextraction (SPME) and analyzed by Gas Chromatography Mass
102 Spectrometry (GC-MS). A 1 ± 0.05 mL or g sample was weighed into a 20 mL vial, with the addition of an
103 internal standard (81.3 ng of 3-pentanone) and closed by a silicone/PTFE septum and an aluminium cap. The

104 samples were then loaded onto a Triplus RSH autosampler (ThermoFisher Scientific, Waltham, MA, USA)
105 for extraction at 37 °C for 15 min, using a divinylbenzene/carboxen/polydimethylsiloxane 50/30 mm SPME
106 fibre assembly (Supelco, Bellefonte, PA, USA). The fibre was desorbed at 220 °C for 2 min in the injection
107 port of the gas chromatograph, operating in the splitless mode. The GC-MS analyses were performed using a
108 Trace1300 gas chromatograph equipped with an ISQ Series 3.2 SP1 mass spectrometer (ThermoFisher
109 Scientific, USA). The compounds were separated on a VFWAX-MS thermo capillary column (60 m, 0.25 mm,
110 0.25 mm, Agilent Technologies, Palo Alto, CA, USA) by using the following conditions: injection port
111 temperature, 220 °C; oven temperatures, 40 °C for 0.1 min then 4 °C min⁻¹ to 140 °C, 10 °C min⁻¹ to 220 °C
112 and a final isothermal for 7.5 min. The mass detector was set at the following conditions: detector voltage,
113 1700 V; source temperature, 250 °C; ionisation energy, 70 eV; scan range 33–200 amu. The tentative
114 identification of the peaks was undertaken by means of software Xcalibur 2.0 (Thermo Fisher Scientific, USA),
115 by matching their spectra with the reference mass spectra of the NIST (National Institute of Standards and
116 Technology) library. A semi-quantitation was performed by the internal standard method, and the amounts
117 were expressed as µg L⁻¹ for the milk and µg kg⁻¹ for the curd and mozzarella samples. The quantitative data
118 were then used to calculate the Odor Activity Value (OAV) of each compound, i.e. the ratio between the
119 concentration found in cheese and the odor threshold value taken from the literature (Natrella, Faccia, Lorenzo,
120 De Palo, & Gambacorta, 2020b). When OAV exceeded level 1, the molecule was considered as potentially
121 aroma-active.

122 2.2.2. *Sensory analysis of cheese*

123 Sensory analysis of the mozzarella samples was conducted by a panel composed of 8 trained assessors
124 belonging to the Italian Association of Cheese Tasters (ONAF). They had been selected following international
125 standard (ISO, 8586-1: 1993), had attended a 20 h course for cheese evaluation (aroma, taste, texture) and had
126 at least a 3-year experience in mozzarella evaluation. The panellists performed a discrimination test by paired
127 comparison with balanced presentation. The two types of samples (control and aseptic) were indistinguishable
128 since they had the same appearance, and were offered in disposable white dishes with three digit code applied
129 on the edge. Since the SA samples contained sodium azide, the test was exclusively based on an olfactory

130 evaluation. The assessors were asked to find eventual differences in the sample pairs and, in the case of
131 perceived difference, to indicate which sample had the higher overall odor intensity.

132 2.2.3. Statistical analysis

133 All samples were analyzed in triplicate. Calculation of the means, analysis of Variance (ANOVA), paired
134 comparison test and PCA were done by using XLSTAT-Sensory software (Addinsoft, France).

135 3. Results and discussion

136 3.1. Gross composition and TBC

137 The three batches of milk presented a similar gross composition, since the fat and protein contents varied in
138 the range 3.61-3.83 and 3.23-3.31, respectively. After the maturation phase, the following TBC values were
139 found: 25,000 colony forming units (cfu) mL⁻¹ (batch coded as HQ); 224,000 cfu mL⁻¹ (batch coded as MQ)
140 and 989,000 cfu mL⁻¹ (batch coded as LQ). The different microbial loads were reflected in different pH values,
141 which varied from a minimum of 6.66 (LQ) to a maximum of 6.79 (HQ). After cheesemaking, the gross
142 compositions of the resulting mozzarella cheeses were not significantly different, with average values of 62.3%
143 $\pm$ 2.3 moisture, 16.5% $\pm$ 1.2 total protein and 17.0 % $\pm$ 1.7 fat.

144 3.2. Total VOC

145 Table 1 shows the results of the VOC analyses on milk, curd, and mozzarella from the three cheesemaking
146 trials (compounds grouped by chemical classes). As expected, in each trial the profiles of the control and
147 aseptic milk were very similar, since the analyses were performed immediately after the addition of the
148 preservative. The only difference regarded the class of acids, whose higher level in aseptic milks depended on
149 the formation of hydrazoic acid deriving from dissociation of sodium azide. Of course, the abundance and
150 complexity of the volatile fraction increased as the microbiological quality of milk got worse. Instead,
151 significant differences were observed in curd and cheese, with relevant variations from trial to trial. In LQ trial,
152 the aseptic curd had a lower content of the total VOC than milk, indicating the loss of some volatiles during
153 curdling, and a higher content than mozzarella, indicating a negative impact exerted by the stretching phase.
154 It was probably due to a “volatilization/solubilization effect” that caused part of the VOC that were present in

155 the curd to volatilize or to dissolve into the stretching water; this result also suggests a poor formation of new
156 volatile compounds upon heating (LAVORO CAGLIATA MIO?). In contrast, the control mozzarella
157 contained more VOC than the curd, and in the light of the results obtained on the aseptic samples, their
158 formation should be ascribed to microbial activity. It is likely that it took place during the cooling phase, since
159 the temperature during stretching should be too high to allow microorganisms to metabolize. As to the effect
160 of the temperature, an influence was observed in both control and aseptic mozzarella. In particular, MozzSA90
161 had an about double concentration than MozzSA70, which was mainly attributable to ketones. The effect was
162 more evident in the control samples, with MozzC70 showing an about 5-fold higher VOC content than
163 MozzC90; the increase regarded almost all chemical groups, but also in this case the most affected compounds
164 were ketones (8-fold more abundant than in MozzC90), followed by alcohols (about 4-folds more abundant).
165 Probably, the lower stretching temperature allowed greater formation of microbial-derived VOC during the
166 cooling phase, since microorganisms underwent to less intense thermal stress.

167 The experimentations conducted on MQ and HQ milks gave some different results with respect to LQ.
168 In both cases, the VOC concentrations in the whole sample sets were generally lower, in agreement with the
169 low TBC values found in the milk, and the changes connected to the presence of microorganism or stretching
170 temperatures did not always follow the same trend. In fact, in both trials the decrease from milk to curd in the
171 aseptic samples was not observed, probably because the effect of curdling was poorly evident at low VOC
172 concentration. It is worth mentioning that in HQ trial even the effect of the preservative sometimes was barely
173 detectable, since the total VOC contents in milks, curds and MozzC70 were not statistically different, as well
174 as were those in MozzC70 and MozzC90. These results clearly indicated that the microbial activity in the
175 control samples was very scarce, in agreement with the low TBC level of milk (25,000 cfu mL⁻¹). Despite of
176 the scarce presence of volatile compounds, the volatilization/solubilization effect exerted by stretching was
177 not only detected in the aseptic samples, but also in one of the control mozzarella (HQ sample stretched with
178 water at 90°C). The effect of the temperature was also observed, both in the aseptic and control samples, but
179 in a less marked way than in LQ. In fact, MozzC70 was again the richest sample in VOC, but the levels
180 observed were 7-fold and 27-fold lower (for MQ and HQ, respectively) than in the previous experimentation;
181 the levels also decreased in MozzC90 (about 2-fold lower for MQ and about 7-fold for HQ). These findings

confirm the relevant impact of milk microbiota on VOC formation in this type of mozzarella, in which the starter microflora is absent.

PCA allowed comparing and summarizing the impact of the experimental variables on VOC formation in the three trials (Figure 2). LQ graph explained 93.43% of the total variance in the whole sample set, with F1 accounting for 80.76% and F2 for 9.68%. A clear separation of MozzC70 was obtained along the positive side of F1, with all loadings oriented towards it. MozzC90 tended to cross F1 towards MozzC70, but remained very far from it, evidencing very well the effect of the stretching temperature in the control samples. The remaining samples laid behind the loadings, however, it must be highlighted that the extremely high VOC content of MozzC70 tended to “flatten” the differences among the other samples, whose clustering was underestimated on the plot. Differently, four clusters were present in MQ graph, one including the control mozzarella samples stretched at 70°C, one other including those stretched at 90°C, a third one the control curd and the last one all other samples. The effect of the stretching temperature in the control experimentation was evident along both F1 (52.52% of the variance explained) and F2 (34.05%), and the cluster formation was mainly due to the concentration of total VOC, ketones, aldehydes and alcohols. With respect to the previous graph, the distance between the two control mozzarella samples decreased, depicting very well the reduction of the effect of the stretching temperature. The effect in the aseptic experimentation was not displayed on the plot due to the poor VOC values. Finally, HQ graph explained only 72.65% of the overall variance along the two principal components, the most important of which accounting for 47.32%. In this experimentation, the scarce contribution of microbial activity to the VOC formation determined a very different pattern. Nevertheless, the effect of the stretching temperature was still visible for both the control and aseptic samples: the former resulted to be better separated along F1, the latter along both components.

3.3. Single volatile compounds, Odor Activity Value and sensory analysis

Overall, 41 different volatile organic compounds were identified in the whole set of samples (results not shown), and the most represented in curd and mozzarella (i.e. exceeding 3 µg kg⁻¹ at least in one type of sample) were acetoin, diacetyl, ethanol, 3-methylbutanal, 3-methyl-1-butanol, 2-butanone, acetic and butanoic acids. These are typical microbial-derived volatile metabolites, even though some of them can also derive from heat-favoured chemical reactions (Bertrand, Meyer, Machado-Maturana, Berdagué, & Kondjoyan, 2015). In

209 general, since they increased from curd to mozzarella in the control trials and were absent or poorly present in
210 the aseptic samples, their formation was ascribed to microbial metabolism. In the control trials, only one
211 compound constantly decreased after the stretching phase, which was acetone, a cheese compound that directly
212 derives from milk, in which it forms throughout cow's metabolism (Clarke et al., 2020; Toso, Procida, &
213 Stefanon, 2002). As to the effect of heat, in the control samples only two compounds increased upon the
214 application of the higher stretching temperature, i.e. 2-methylbutanal and nonanal. The former also increased
215 in the aseptic samples in which the increase also regarded 2-butanone, hexanal and hexanoic acid. For
216 aldehydes, it is known that in dairy products they can derive from oxidative reactions (Ianni, Bennato, Martino,
217 Grotta, & Martino, 2020), which in the present experimentation could have been accelerated by the highest
218 stretching temperature, possibly in synergy with higher redox potential determined by the absence of microbial
219 activity. It is worth mentioning that hexanal is widely used as indicator of oxidation in different types of foods
220 (Andersson & Lingnert, 1998; Christensen & Hølmer, 1996; Shahidi & Pegg, 1994; Sunesen, Lund, Sørensen,
221 & Hølmer, 2002). The formation of new volatile compounds exclusively attributable to the application of heat
222 during stretching could not be observed in the control sample due to overlapping of the microbial activity,
223 thus, the data of aseptic curds and cheeses were carefully deepened, even taking into consideration the
224 compounds detected at trace level. Indeed, three possible newly formed molecules were identified (2-
225 butoxyethanol, 6-methyl-5-hepten-2-one and furfural), which also reached a higher concentration in the cheese
226 stretched with water at 90°C. Unfortunately, they were not detected in all trials and the concentrations were
227 always very low (much less than 1 $\mu\text{g}\cdot\text{kg}^{-1}$). Furfural and 2-butoxyethanol have been sometimes associated to
228 heat treatments in dairy products, and the former is considered a possible indicator of the Maillard reaction
229 under mild heating condition (Forss, 1971; Shiratsuchi, Yoshimura, Shimoda, Noda, Osajima, 1995; Xing et
230 al., 2020). Xing et (2020) reported a furfural content of 16 $\mu\text{g}\cdot\text{kg}^{-1}$ in pasteurized milk treated at 70 °C/15 sec,
231 whereas the maximum level measured in the present study was 0.27 $\mu\text{g}\cdot\text{kg}^{-1}$. Such a low value, together with
232 the absence in one of the three trials, does not allow to establish with certainty that its formation was ascribable
233 to the stretching process. On the other hand, it cannot be excluded that the time/temperature conditions applied
234 during curd stretching sometimes triggering the reaction.

235 The OAV calculation of LQ samples (Table 2) demonstrated that MozzC70 had the highest number of
236 theoretically aroma-active compounds, which were diacetyl, 2- and 3-methylbutanal, nonanal, ethanol, 3-
237 methyl-1-butanol, acetic acid and 3-methylbutanoic acid. Among them, diacetyl showed the highest OAV
238 (3078.4), followed by 3-methylbutanal (739) and 2-methylbutanal (332); they are responsible of buttery, fruity
239 and apple note, respectively. MozzC90 was the second sample as to the number of aroma-active compounds
240 (5 versus 8 of MozzC70), with 3-methylbutanal and 2-methylbutanal having OAV of 321.5 and 128
241 respectively. The aseptic samples had only three potentially aroma-active compounds, of which two were in
242 common (2-methylbutanal and nonanal) and one was different (3-methylbutanal for MozzSA70 and acetic
243 acid for MozzSA90). Their values were much lower than those found in the control cheeses, since the highest
244 level was that of 2-methylbutanal in MozzSA90 (99.1). OAV data of MQ experimentation confirmed that the
245 control samples had a higher number of VOC with OAV above 1, but the number decreased (they were only
246 5 versus 8 in LQ); in contrast, the number did not change for the aseptic samples (3). Again, the highest OAV
247 were detected in MozzC70, with 3-methylbutanal reaching the highest level (338.2). As already observed for
248 LQ, aldehydes resulted potentially to primary contribute to the aroma of the aseptic samples, in particular
249 heptanal, octanal and nonanal for MozzSA70 and nonanal, octanal for MozzSA90 (plus acetic acid). Finally,
250 also in the data from HQ trial MozzC70 seemed to be the sample containing the highest number of VOC with
251 OAV>1, followed by MozzC90, MozzSA70 and MozzSA90; however, the values were much lower with
252 respect to the other trials.

253 Figure 3 shows the results of the paired comparison test only for LQ and MQ mozzarella, since the panellists
254 were not able to find differences among the samples from HQ experimentation. In both trials, the results
255 matched well with OAV calculation, since: i) the control samples resulted to have higher odor intensity than
256 the aseptic ones, and MozzC70 always received the highest score; ii) the odor intensity of the aseptic samples
257 stretched with water at 90°C was higher than that of the cheeses stretched at 70°C. The absence of differences
258 in the sensory characteristics of HQ mozzarella samples indicated that the panellists were not able to capture
259 the effect exerted by both the preservative and the stretching temperature that, instead, was grasped by the
260 instrumental analysis.

261 4. Conclusions

262 From the results of the experimentation, two main conclusions can be drawn. The first is that, predictably, in
263 high moisture mozzarella obtained by direct acidification, the better is the microbiological quality of milk, the
264 lower is the VOC content and the aroma intensity. The second relevant conclusion is that the impact of the
265 stretching temperature on the cheese odor is negative, since the higher is the temperature the greater is the loss
266 of VOC due to volatilization and/or solubilisation in the stretching water. Consequently, the effect becomes
267 negligible when the curd is poor in VOC, as normally happens when pasteurized milk is used. Several possible
268 practical applications can derive from the present investigation. The first one is for traditional cheesemakers
269 who commonly produce the cheese from unpasteurized milk; in this case, the application of a maturation phase
270 to the cheese milk before starting the manufacturing process should improve the sensory quality. Of course,
271 the hygienic aspects needs to be carefully kept under control to validate the procedure. A second possible
272 application is that maximizing the stretching parameters could be useful to eliminate possible off-flavors
273 present in the curd. On the other hand, minimizing them should be the rule in industrial mozzarella
274 manufacturing that normally contemplates the use of pasteurized milk. Finally, it can be concluded that the
275 time/temperature conditions applied during the stretching phase of mozzarella seems to be too mild to induce
276 the formation of relevant amounts of new volatile compounds from heat-induced chemical reactions.

277

278 5. References

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360 **Captions to figures**

361 Figure 1. Experimental design applied in the model study.

362 Figure 2. Principal component analysis performed on total VOC of the trials performed on low quality
363 (LQ), medium quality (MQ) and high quality (HQ) milk.

364 Figure 3. Results of the paired comparison test on mozzarella samples deriving from the trials on low
365 quality (LQ) and medium quality (MQ) milk. Significance is indicated in the graph as an asterisk over
366 brackets; the upper brackets refer to the difference between control and aseptic samples, the lower to
367 the difference between samples of the same group (* <0.05; **<0.001). The trials cannot be compared
368 and must be considered separately.

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