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1 **Natural levels of nitrites and nitrates in San Daniele dry cured ham PDO, and in meat, salt**
2 **and sugna used for its production**

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19 **Summary**

20 The aim of the study was to determine the level of the “natural” nitrite and nitrate concentration in
21 raw meat, salt and *sugna* (soft pork fat) used to produce San Daniele dry cured ham (SDDCH) and
22 in SDDCH (PDO) that has been ripened over 14 months under controlled environmental conditions.

23 The average natural nitrite content in meat, salt, *sugna* and dry cured ham was approximately 2, 1, 5
24 and 1 mg/kg, respectively. The natural nitrate content was 8, 6, 8 and 4 mg/kg. Data allowed to
25 determine threshold value for both compounds: the nitrite and nitrate concentrations in San Daniele
26 PDO ham must be considered “natural and not intentional added” when they are less than 4 and 22
27 mg/kg, respectively.

28 The underlying aim of the research was to enable producers to prove no additives were deliberately
29 added during the ham production and to help authorities to identify SDDCH not compliant with the
30 rules.

31
32 **Key words:** Nitrite, Nitrate, Natural Concentration, Threshold Value.

34 **Introduction**

35 San Daniele dry cured ham (SDDCH) is a typical meat product, made with Italian pork (thigh),
36 which is salted and ripened for at least 13 months (Comi & Iacumin, 2012), when it reaches a
37 particular delicate aroma and flavor (Kim *et al.*, 2016; Neethling *et al.*, 2016; Comi & Iacumin,
38 2012). In many areas of the world its popularity is consistently increasing. Italy has promoted the
39 organic protection of this product since 1970, and in 1990 approved a new protection law - No. 30
40 (February, 14th 1990; Denominazione di origine del prosciutto di San Daniele, GU n. 45 del
41 23.2. 1990). Subsequently, the European Union registered the San Daniele ham as PDO with Reg.
42 (CE) n. 1107/1996. Currently, the Reg. (UE) n. 1151/2012, establishing community protection for
43 agri-food products with a designation of origin, has reinforced, adapted and developed schemes to
44 identify quality of European products and foods. The processing phases, which derive from ancient

artisan tradition, are reported in the PDO regulations and in the Ministerial Decree of February, 16 1993, No. 298. The processing stages consist of: the choice of meat, cooling, trimming, massaging, salting, pressing, pre-ripening and rest, tempering and washing, drying, pre-ripening and ripening (Comi & Cattaneo, 2007). The fresh thighs of heavy pig (150-180 kg) include the “zampetto” (foot), as codified in art. 25, Co. 1 of Law n. 30/1990, which is left on the ripened product and constitutes one of the characteristics of the SDDCH. The SDDCH Consortium monitors the most suitable thighs and applies a pre-mark and the complete date of production start (Comi & Iacumin, 2005, 2012). The thighs are then processed and salt and the dehydration/ripening phases are the only parameters influencing their stability and safety. Nitrite and nitrate which are commonly used to produce and maintain the red color of meat, to produce characteristic flavors, prevent fat oxidation and the development of pathogenic and spoilage microorganisms in order to improve the organoleptic, sensorial and hedonic characteristics in cooked and dry cured meats over time (Comi & Iacumin, 2012; Comi & Cattaneo, 2007; Toldrá, 2007), are not allowed to be used in SSDCH. Salt is the only other ingredient permitted to achieve stability and safety.

In dry cured ham, the loss of moisture, ripening and salt prevent the development of any spoilage or pathogenic microorganisms (Comi & Iacumin, 2012, 2005; Comi & Cattaneo, 2007). The lack of nitrites does not affect the typical red color of the meat, which remains stable because of the negative redox potential of the meat.

Given that nitrates have been detected in some commercially PDO SDDCHs, the aim of our work was to verify if this undue presence could be attributable to raw meat and to the other permitted ingredients. So, we determined both in SDDCH ripened over 14 months and in the only ingredients used in the production of SDDCH, meat, salt and *sugna*, the levels of nitrites and nitrates. *Sugna* is the typical paste prepared with rice flour, soft pork fat, and ground peppercorn (pepper), spread on the muscular area not covered by rind, which promotes homogeneous dehydration during the last phase of the production of San Daniele dry cured ham. An additional purpose was to define the threshold values of nitrite and nitrate concentrations naturally present, and to identify any deviations

71 from the permitted standards. This is because, over the years and especially at the level of foreign
72 markets, hams sold as "SDDCH" sometimes showed to have similar levels of both compounds to
73 those of sausages in which their use is allowed.

74

75 **Materials and methods**

76 The natural content of nitrites and nitrates in SDDCH processed according to the strict protocol of
77 the SDDCH Consortium and in the ingredients used in its production was determined. 'Natural'
78 means that both compounds were not deliberately added by the producers during the ham
79 production. The analyzed samples were strictly taken from PDO branded hams ripened over 13
80 months of different companies located in San Daniele (a municipality in north east Italy).

81 The investigated samples thus included:

82 50 slices (about 500 g each) corresponding to fifty SDDCH hams of different ripening times (22
83 hams with a ripening period of 14 months, 10 of 15 months, 10 of 16 months, 7 of 17 months and 1
84 of 19 months).

85 50 samples (about 500 g each) of pork meat derived from thighs used in SDDCH production;

86 3 samples of *sugna* (about 300 g each);

87 10 samples of food salt (300 g each) taken from different production facilities and belonging to 10
88 different lots.

89 Moisture was determined in meat and SDDCH samples according to AOAC (1995) in order to
90 express the nitrites and nitrates concentrations on dry weight. Aw was determined on the SDDCH
91 samples, to verify the conformity of the product to the Consortium standards, using an AquaLab
92 CX-2 Steroglass (Pullman, WA, USA).

93 The nitrite and nitrate detection was carried out following Mirna and Schutz (1972), modified
94 according to AOAC (1990). This method is widely used for the determination of nitrites and nitrates
95 in food products (meat and meat products, milk and cheese, vegetables, and drinking and waste
96 water). It is more productive than other colorimetric methods because it has a detection limit in

97 meat of 0.05 mg/kg. The United States Environmental Protection Agency (EPA, 1993) recommends
98 a similar method for nitrite and nitrate determination based on a colorimetric reaction using a
99 cadmium reduction column, which has been validated by various international standardization
100 organizations (Table 1). It is a spectrophotometric method, which is therefore cheap, easy to use,
101 does not require special or expensive equipment (such as HPLC) and can be applied to various
102 matrices (plants, meat products, baby foods, dairy products and surface waters).
103 In brief, the method involves a hot extraction of the sample with water, which prevents any
104 interference due to ascorbic acid or other reducing agents. Subsequently, as stated by EPA (1993)
105 "the sample is filtered and passed through a column containing granulated copper - cadmium to
106 reduce nitrate to nitrite. The nitrite (that was originally present plus reduced nitrate) is determined
107 by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine
108 dihydrochloride to form a highly colored azo dye which is measured colorimetrically at 540 nm.
109 Separate, rather than combined nitrate-nitrite, values are readily obtained by carrying out the
110 procedure first with, and then without, the Cu-Cd reduction step". [SEP]A calibration curve is also
111 periodically performed. Nitrites and nitrates were expressed in mg/kg as sodium nitrite and sodium
112 nitrate.

113

114 **Results**

115 The results of the moisture determination in meat, ham and *sugna* samples are summarized in Table
116 2. These values were investigated in order to formulate the nitrite and nitrate concentrations with
117 respect to dry weight. Figures 1 and 2 show the percentage of moisture and the A_w correlated to the
118 ripening period. As shown, all the samples had A_w levels lower than 0.92. The moisture values
119 were variable and were not correlated to the different ripening ages. All were below the maximum
120 limit allowed by the SDDCH Consortium (63%).

121 Salt and *sugna* can naturally contain nitrites and nitrates, the former as impurity, the latter mainly
122 due to ground peppercorns, and consequently can potentially "contaminate" meat and hams.

123 Therefore, the presence of both compounds was investigated in three *sugna* and in ten salt samples,
124 obtained from different ham factories (Tables 3 and 4). In *sugna*, the average nitrite and nitrate
125 concentrations were 5 ± 0.30 mg/kg and 8 ± 5.2 mg/kg, respectively; nitrates showed a greater
126 variability compared to nitrites. In fact, the average nitrate concentration varied from a minimum of
127 4 to a maximum of 14 mg/kg (Table 3). The concentrations of nitrites and nitrates in food salt were
128 also low (Table 4), on average 1 ± 0.8 mg/kg and 6 ± 2.9 mg/kg, respectively. Therefore, the
129 contribution of salt to the concentrations of both compounds in SDDCH was theoretically very low,
130 even considering that at the end of ripening the salt concentration can vary from 4.4% to 7.1%, as
131 calculated on the basis of the limit values of the salt/humidity ratio imposed by PDO legislation.

132 The average concentrations of natural nitrites and nitrates of fresh meat were 2 ± 0.9 mg/kg and $8 \pm$
133 3.4 mg/kg, respectively; the range was quite wide (Table 5). Finally, the average nitrite and nitrate
134 concentrations in SDDCH were 1 ± 0.5 mg/kg and 4 ± 3.1 mg/kg, respectively (Table 6).
135 Furthermore, the values found in the 50 samples of SDDCH were lower than those observed in
136 fresh meat.

137 This could be explained by the fact that the analyzed meat was not the same as that used in the
138 production of the sampled SDDCH and that the natural levels depend on different factors such as
139 feeding, water and farming conditions. Moreover, it could be hypothesized that during SDDCH
140 production, some nitrites and nitrates are lost when exudates and residual blood drip from the meat
141 surface, and some link to myoglobin to form nitrosyl-myoglobin.

142 The values obtained in fresh meat are also similar to those determined in farmed salmon meat (1-2
143 mg/kg of nitrites and 4-6 mg/kg of nitrates, unpublished data). Given that the ingredients can
144 provide, albeit minimally, nitrites and nitrates, that their content can be concentrated by dehydration
145 and that the loss of water and exudates can partially eliminate them, we thus investigated the
146 threshold values of natural (non-added) nitrite and nitrate.

147 Table 6 shows the variability of nitrite and nitrate concentrations, observed in SDDCH. The data

148 include minimum and maximum values, mean, median, standard deviations and fiducial confidence
149 intervals of the means ($p < 0.05$ and $p < 0.01$). The maximum limit of the fiducial interval is about 1
150 mg/kg for nitrites in SDDCH ($p < 0.01$) and is below the maximum value observed in the 50
151 SDDCH samples (2 mg/kg). For nitrates, the maximum limit of the fiducial interval is about 4
152 mg/kg ($p < 0.01$) and it is below the maximum value observed in the 50 SDDCH samples (11
153 mg/kg).

154 We thus decided to double the observed maximum values and to consider them as threshold values.
155 The threshold values, we proposed, appear to be mostly below the levels found in some counterfeit
156 raw hams products of unknown origin and marked as SDDCH PDO, collected worldwide by the
157 SDDCH Consortium. Therefore, accepting these threshold values, there is no risk of negatively
158 judging either raw meat or SDDCH products with concentrations higher than the nitrite and nitrate
159 average values, due to natural variability. Irrespectively of the ripening time and the concentration
160 ranges of both compounds in the SDDCH samples, a SDDCH could be identified as PDO when the
161 nitrite and nitrate concentrations do not exceed 4 mg/kg and 22 mg/kg, respectively (Table 7). This
162 table shows the nitrite and nitrate threshold values proposed for raw meat, *sugna*, and salt, allowing
163 producers to test and accept ingredients for SDDCH PDO production.

164

165 **4. DISCUSSION**

166 Dry Cured Ham is one of the main meat products obtained with a wide variety of ingredients and
167 technologies, which influence texture and aroma. Nitrites and nitrates represent the main
168 preservatives of meat products, but for SDDCH production their use is forbidden. However, their
169 presence has often been highlighted at levels of 60-70 mg/kg in several dry cured hams labelled
170 "SDDCH PDO". The aim of the work was thus to verify the natural concentration of such
171 substances in raw materials (meat, salt), technological adjuvants (*sugna*) used to produce SDDCH
172 and in SDDCH ripened over 13 months.

173 During the dehydration and ripening phases, a slow but progressive loss of moisture, A_w reduction

174 and increase of the salt concentration are observed, which stabilize the ham (Comi & Iacumin,
175 2012) and influence the enzymatic activity and consequently the sensorial characteristics of the
176 final product (Jiménez-Colmenero *et al.*, 2011). A wide Aw and moisture variability is observed in
177 the tested SDDCH, not dependent on the ripening times. In fact, various hams with different drying
178 times, ranging from 14 to 19 months, have the same Aw and moisture. This is due to differences in
179 the raw meat and in the processing conditions applied, which may vary between one production
180 facility site and another, even if the same protocol is applied. However, this large variability does
181 not affect the wholesomeness and the stability of the hams (Cviková *et al.*, 2016; Kunová *et al.*,
182 2015; Parolari *et al.*, 2009). In particular, regardless of the ripening time, the Aw of the tested hams
183 was less than 0.92. For this reason, they should be considered as being healthy, edible and
184 complying with the SDDCH Consortium rules (Comi & Iacumin, 2012; Comi & Cattaneo, 2007).

185 Salt is the key ingredient for the production of hams. In fact, salting is the first step in ham
186 production (Martínez-Onandi *et al.*, 2016) and in particular in SDDCH. Sea salt, medium-grain wet,
187 is used without the addition of other ingredients such as nitrite and nitrate. The production
188 specifications of the PDO only include salt without nitrite. Salt inhibits the development of spoilage
189 and/or pathogenic microorganisms and solubilizes the soluble salt proteins, which are then degraded
190 by the tissue enzymes responsible for ripening. In fact, salt activates these enzymes and especially
191 cathepsins D (Toldrá, 2007).^[11]_[SEP]

192 However, salt can contain nitrite and nitrate as an impurity, but our data showed that the presence of
193 nitrite and nitrate impurities in salt does not affect their concentration in the final product. In fact,
194 considering the levels of the two compounds and the percentage of salt in the SDDCH (up to 6-7%),
195 salt appears to increase the nitrite and nitrate concentration of about 0.1 mg/kg and 0.4 mg/kg,
196 respectively. This increase is not significant respect to the natural levels of nitrite and nitrate of pork
197 meat. However, it was important to find a threshold value for the concentration of nitrites and
198 nitrates for salt as well. This value is 6 mg/kg and 24 mg/kg, respectively.

199 A threshold is also needed for *sugna*. Our results revealed in *sugna* a low content of both

200 compounds, but higher than in salt. However, the contribution of *sugna* is less than that of salt
201 because the contact between *sugna* and the ham is only on the muscular area not covered by rind
202 and occurs in a phase where *A_w* is low and diffusion is slow. For *sugna*, we established a threshold
203 of 10 ppm for nitrite, and 28 ppm for nitrate.

204 Fresh meat may contain natural nitrites and nitrates (Iammarino *et al.*, 2013; Iammarino & Di
205 Taranto, 2012), as well as meat products treated only with salt and sugar and organic meat products
206 not treated with nitrate substitutes (Sebranek & Bacus, 2007.) Data found about the presence of
207 both the additives in the investigated fresh meats were similar to those obtained by Sebranek &
208 Bacus (2007) and very lower compared to those found in meat products treated with nitrates and
209 nitrites by various authors (Armenteros *et al.* 2012; Cantoni & Bianchi Paleari, 1980).

210 The natural origin of nitrites and nitrates in meat is due to the nitrogen metabolism of the animal
211 and the feed. In mammals, nitric oxide derives from the degradation of arginine through the action
212 of the enzyme NO-synthase (Hibbs *et al.*, 1992). In the presence of oxidized haemoglobin or the
213 enzyme superoxide dismutase (Benjamin & Collins, 2003) the nitrogen oxide is then oxidized at
214 cellular level to nitrite or nitrate, and these are eliminated via urine or faeces and/or partly retained
215 in the body. In fact, humans and animals eliminate more nitrate than they actually ingest (Mitchell
216 *et al.*, 1916). Nitrites can also derive from vegetables, used for feeding pigs once ingested, the
217 nitrates are reduced by bacteria, saliva or by endogenous nitrate reductase into nitrite (Li *et al.*,
218 1997).

219 Nitrite and nitrate are also ingested directly in this form with feed and food. During the vegetables
220 preservation, bacteria, such as Staphylococci, Micrococci and Streptococci, grow and reduce nitrate
221 to nitrite (Benjamin & Collins, 2003; Li *et al.*,1997). This explains why nitrite and nitrate were
222 found in fresh meat used for SDDCH production. Thus, according to our data, it was possible to
223 suggest a threshold limit for nitrite and nitrate in pork meat suitable for SDDCH production;
224 consequently, the PDO SDDCH producers can accept fresh meat, when the nitrite concentration is
225 below the threshold values of 14 mg/kg and nitrates below 42 mg/kg. In this case, the threshold

values expressed on the dry weight (44 mg/kg for nitrites and 166 mg/kg for nitrates) may also be useful to avoid disputes related to the degree of meat moisture. In fact, moisture can vary due to fat content and pig genetics, slaughtering techniques, ageing, time and temperature of meat storage before salting. A similar admissible threshold value of about 30 mg/kg of nitrates in fresh pork or bovine meat and of 40 mg/kg in fresh horse meat were also suggested by other Italian researchers (Iammarino et al., 2013; Iammarino and Di Taranto, 2012).

The most important part of our work concerned the evaluation of the "natural" concentration of nitrites and nitrates in SDDCH. The aim was to discover threshold levels of their presence in order to prevent any illegal additions. In this regard, to avoid discussions concerning the correlation of the concentration of such compounds and the level of ripening of the ham, we formulated a threshold value. Beyond this value, the determined levels can be considered as not having a natural origin, but rather the result of an intentional addition or, in the case of salt and *sugna*, not suitable for use in this production.

Our proposal is in line with several authors who have extensively studied the evolution of nitrites and nitrates in cured meat during its ripening (Sebranek & Bacus, 2007). It has been reported that after an addition of nitrate in concentration of 150 mg/kg to pork and/or bovine meat, a clear decrease of the nitrate is observed by its reduction to nitrite. In any case, the average residual concentration of both is higher than the "natural" concentration. In particular, after their addition, the values found are always higher than 40 mg/kg (nitrite) and 26 mg/kg (nitrate) (Cantoni & Paleari, 1980). This residual concentration can increase when the initial addition is higher than 150 mg/kg of nitrate or equal to 250 mg/kg of a mixture of nitrite and nitrate (Hospital *et al.*, 2017; Armenteros *et al.*, 2012; Comi *et al.*, 2005).

Finally, the method used for the determination of nitrite and nitrate was proven to be valid, efficient, inexpensive, simple and easy to apply and it is recommended by various International Standard Method Organization (EPA, 1993; ISO, 2006; AOAC, 1995).

252 **5. Conclusions**

253 The data obtained highlight that the concentration of nitrite and nitrate in SDDCH PDO must be
254 considered natural when it is, respectively, less than 4 and 22 mg/kg of the ripened product,
255 irrespectively of the ripening time (14-19 months).

256 The threshold values can be used to determine when SDDCH can legitimately receive the PDO
257 mark or alternatively be designated as Dry Cured Ham (national, foreign ham, etc.).

258 In our opinion, there is in any case no need to add nitrite or nitrate salts in SDDCH production,
259 because salting at refrigeration temperature, dehydration and subsequent ripening already provide a
260 stable product characterized by low A_w (≤ 0.92) and a uniform and widely acceptable color.

261 We also suggest to accept raw meat with nitrite and nitrate concentrations below the threshold
262 values of 14 mg/kg and below 42 mg/kg, respectively. The threshold values expressed on dry
263 weight (44 mg/kg for nitrite and 166 mg/kg for nitrate) may also be useful to avoid disputes related
264 to the degree of moisture of the meat.

265

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269

270 **Conflict of interest**^[1]_{SEP}

271 The authors declare that they have no conflict of interest.

272 .

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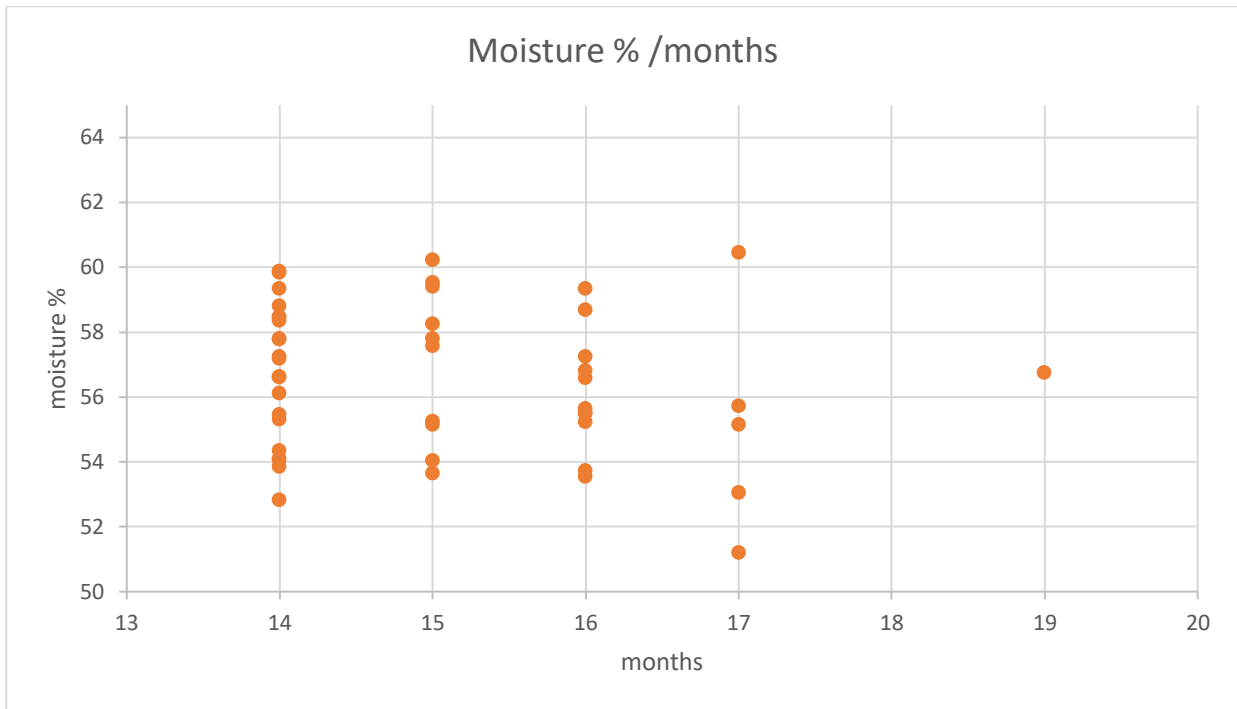
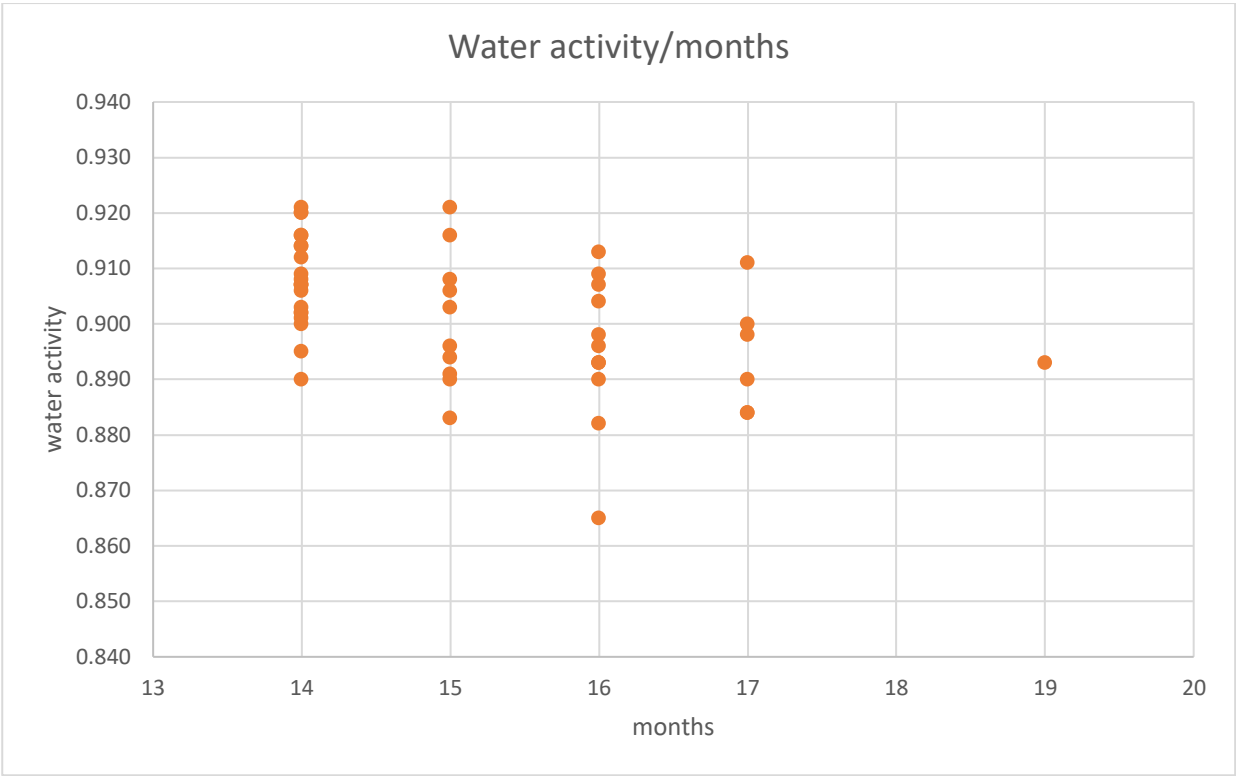


Figure 1: Correlation between moisture and ripening time of dry cured ham

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379 Figure 2: Correlation between water activity and ripening times of dry cured ham

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393 Table 1: International standard methods for nitrites and nitrate detection based on Griess reaction
 394 using a cadmium reduction column (LO Man-fung, 2008, modified,
 395 <https://www.govtlab.gov.hk/g/texchange/sudan.pdf>.)
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Standard	Samples NO ₂ /NO ₃
ISO 2918/1975 (E) – 3091/1975 (E) – Determination of nitrates, reference method	Meat and meat products
ISO (2003/2006)	Milk and milk products
EPA, (1993)	Various food products
AOAC, (1990)	Cheese
AOAC (1995)	Meat – Food for Infants

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Table 2: Mean, range and standard deviation of moisture in meat, dry cured ham and sugna (%)

Moisture %	Meat	Dry Cured Ham	Sugna
Mean	73.0 ± 2.1	56.4 ± 2.4	4.3 ± 0.5
Range	66,2-84.2	49.8 – 60.4	4.0 – 4,9
Sample number	50	50	3

Legend: Mean ± standard deviation

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Table 3: Nitrites and nitrates in sugna (mg/kg)

Value	Nitrites WW	Nitrites DW	Nitrates WW	Nitrates DW
Mean	5 ± 0.3	5 ± 0.3	8 ± 5.2	9 ± 5.4
Range	4-5	4-5	4-14	4-14
Sample number	3	3	3	3

Legend: Mean ± standard deviation; Wet Weight (WW), Dry Weight (DW)

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Table 4: Nitrites and nitrates in Salt (mg/kg)

Value	Nitrites	Nitrates
Mean	1 ± 0.8	6 ± 2.9
Range	< 1-3	2-12
Sample number	10	10

Legend: Mean ± standard deviation

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Table 5: Nitrites and Nitrates in fresh meat (mg/kg)

Value	Nitrites WW	Nitrites DW	Nitrates WW	Nitrates DW
Mean	2 ± 0.9	7 ± 3.2	8 ± 3.4	32 ± 15.1
Range	<1-7	1-22	1-21	3-83
Median	2	7	8	31
Sample number	50	50	50	50
95% confidence interval	2 ± 0.3	7 ± 0.9	8 ± 1.0	32 ± 4.2
99% confidence interval	2 ± 0.3	7 ± 1.2	8 ± 1.2	32 ± 5.5

Legend: Mean ± standard deviation; WW - Wet Weight; DW - Dry Weight DW); 95% and 99% confidence intervals of the means.

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Table 6: Nitrites and Nitrates in San Daniele Dry Cured Ham (mg/kg)

Values	nitrites WW	nitrites DW	nitrates WW	nitrates DW
Mean	1 ± 0.5	2 ± 1.3	4 ± 3.1	9 ± 7.1
Range	<1-2	< 1-5	<1-11	<1-26
Median	1	2	4	9
Samples number	50	50	50	50
95% confidence interval	1 ± 0.2	2 ± 0.4	4 ± 0.9	9 ± 2.0
99% confidence interval	1 ± 0.2	2 ± 0.5	4 ± 1.2	9 ± 2.6

Legend: Mean ± standard deviation; WW - Wet Weight; DW - Dry Weight DW); 95% and 99% confidence intervals of the means.

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Table 7: Nitrites and Nitrates threshold value (mg/kg)

Product	nitrites WW	nitrites DW	nitrates WW	nitrates DW
Fresh meat	14	44	42	166
Dry cured ham	4	10	22	52
Salt	6	6	24	24
Sugna	10	10	28	28

Legend: WW - Wet Weight; DW - Dry Weight DW

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