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# RADIO-FREQUENCY AND OPTICAL TECHNIQUES FOR ANCHOVY FRESHNESS EVALUATION

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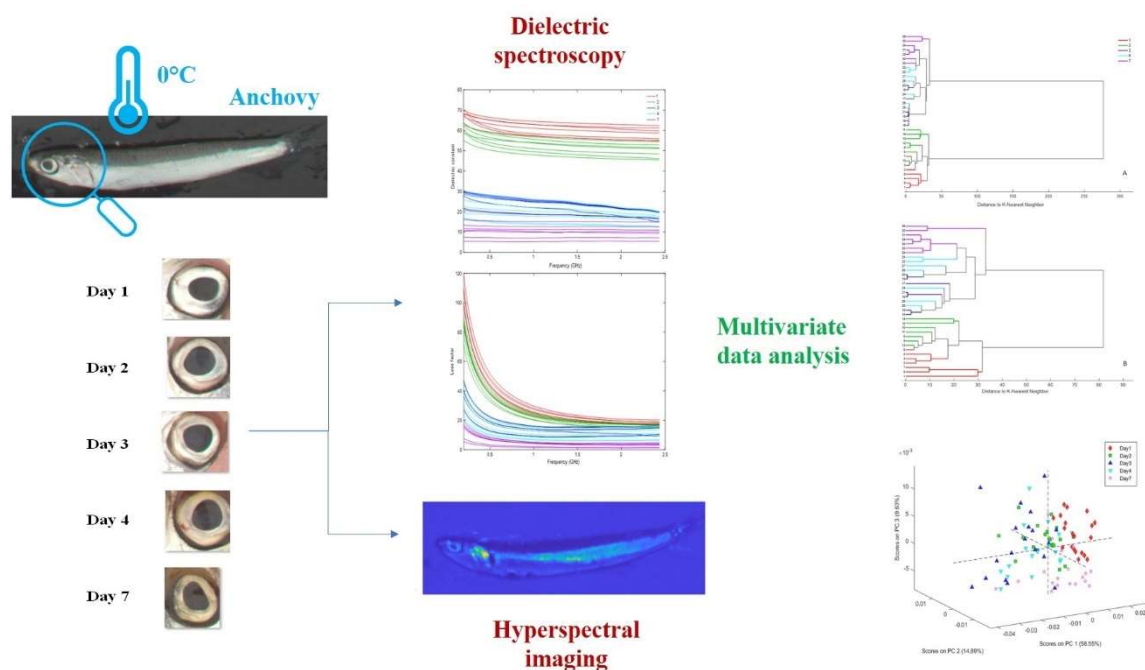
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## Abstract

The freshness of anchovies (*Engraulis Encrasicolus*) was estimated by Radio-Frequency (RF) and optical techniques in order to propose non-destructive, and objective methods for rapid screening. Measurements were conducted until 7 days at 0°C, storing the fishes under ice. Image analysis,

21 dielectric spectroscopy and hyperspectral images were conducted on fish eyes while mechanical  
22 properties were measured on fish vomit. Evaluation of images by RGB scale highlights differences  
23 due to ageing. Maximum force decreases as a function of degradation process time. Cluster analysis  
24 on dielectric spectra and 3D-PCA on hyperspectral images of fish eyes revealed a good ability to  
25 characterize ageing modification presenting suitable methods for the development of a non-  
26 destructive, and rapid system for evaluation of fish freshness.

27

28 **Keywords:** anchovy freshness, multivariate analysis, dielectric spectroscopy, hyperspectral  
29 imaging, non-destructive methods, fish eyes

## 30 **Introduction**

31 Mediterranean anchovy (*Engraulis encrasicolus*) plays a key role both in the human diet, thanks to  
32 the high presence of protein and n-3 fatty acids in its meat (Gencbay & Turhan, 2016), and in the  
33 economy of the Mediterranean countries. An average of 270,000 tons are landed every year in the  
34 whole Mediterranean basin (FAO, 2018). The most important aspect to consider and monitor, not  
35 only in anchovies, but in the whole fish industry and commercialization, is freshness: after a brief  
36 period of time, dead fishes show an increase in the growth and activity of microorganism and in the  
37 oxidation of lipids (Erkan, Özden, Alakavuk, Yildirim, & Inuğur, 2006). These two processes cause  
38 a fast deterioration of critical quality parameters for the consumers, like appearance, odor and taste  
39 (Kyranas & Lougovois, 2002; Olafsdottir, Jonsdottir, Lauzon, Luten, & Kristbergsson, 2005). The  
40 speed and severity of these changes are highly variable, depending on several parameters like species,  
41 fat content, storage conditions and temperature (Alasalvar, Taylor, Öksüz, Shahidi, & Alexis, 2002;  
42 Ashie, Smith, & Simpson, 1996; Olafsdottir et al., 2004). Moreover, the refrigeration process does  
43 not stop the whole microbial activity, thanks to the presence of psychotropic bacteria (Pedrosa-  
44 Menabrito & Regenstein, 1988). The traditional method to assess fish freshness is the sensory  
45 inspection, used by the European Union since the '70 (CEE, 1976). Nowadays, the most common  
46 and widespread sensory method is undoubtedly the Quality Index Method (QIM), based on evaluating

the body parts of the fish changing during the decaying process, like eyes, gill, and skin. This method is very specific, because a different evaluation table is created for every fish species (Pons-Sánchez-Cascado, Vidal-Carou, Nunes, & Veciana-Nogués, 2006). Regarding anchovies, several papers focus on the development of QIM schemes (Massa, Manca, & Yeannes, 2012; Pons-Sánchez-Cascado et al., 2006), or use them to assess changes (e. g. increase of Total Volatile Basic Nitrogen (TVBN), determination of cholesterol oxides or impact of natural plant extracts) in the fish samples under various conditions (Bensid, Ucar, Bendeddouche, & Özogul, 2014; Marrone et al., 2012; Özogul, Tugce Aksun, Öztekin, & Lorenzo, 2017). However, these methods have some disadvantages: they are time-consuming and require high skilled operator, making it difficult to use them for in-line or on-line industrial applications. To overcome this, in the last decades several indirect and non-destructive techniques were developed to assess physical and chemical parameters related to fish freshness, going from the use of biosensors (Draisci et al., 1998) and electronic nose to various spectroscopic analyses (Varrà, Ghidini, Ianieri, & Zanardi, 2021; Velioğlu, Temiz, & Boyaci, 2015). Among the latter, techniques that operate in the visible and near-infrared range (VIS/NIR) are of particular importance, because they allow detecting the vibrations of C-H, O-H and N-H groups (Pu, Feng, & Sun, 2015). In the last years, VIS/NIR devices were used to assess several different parameters, like cold storage time of salmon (Wu, Zhong, & Yang, 2018), trimethylamine concentration and K-value in silver carp (Agyekum et al., 2020, 2019), and discrimination between fresh/thawed Atlantic mullets (Alamprese & Casiraghi, 2015). An evolution of these infrared spectroscopy techniques is represented by Hyperspectral Imaging (HSI): thanks to special cameras, a whole electromagnetic spectrum is acquired for every pixel of an image, providing qualitative and spatial data at the same time. Good results were obtained for a wide variety of species: Ivorra et al. (2016) developed a model for shelf-life prediction of salmon, Cheng et al. (2017) evaluated the K-value in grass carp and silver carp, Khoshnoudi-Nia and Moosavi-Nasab (2019) assessed the values of Total Volatile Base Nitrogen (TVB-N), Psychotropic Plate Count (PPC) and sensory score in rainbow trout fillets, Franceschelli et al. (2020) explored the use of HSI for sardines freshness

73 monitoring. Also the study of more straightforward RGB images (usually focusing on eyes and gills)  
74 represents an active field, as evidenced by recent works on common carp and goldfish (Bachrun Alim,  
75 Suhaeli Fahmi, Purnamayati, & Agustini, 2020; Negi, Yadav, Rawat, & Singh, 2019) and rainbow  
76 trout (Mohammadi Lalabadi, Sadeghi, & Mireei, 2020).

77 A different approach is represented by dielectric analysis, focusing on the acquisition of parameters  
78 like the dielectric constant and the loss factor, influenced, as well as freshness, by moisture (Cataldo,  
79 Piuzzi, Cannazza, & De Benedetto, 2009), physical/chemical changes (Ragni et al., 2017, 2016) and  
80 water activity (Iaccheri et al., 2015). However, in the literature there are only a few examples  
81 regarding fishes: several variables related to freshness were assessed by Kent et al. (2004), thanks to  
82 the use of a coaxial probe, on cod, hake and salmon; Wang et al. (2008) studied the effects of changes  
83 in temperature between 20 °C–120° C, monitoring five different frequencies (27, 40, 433, 915, 1800  
84 MHz) with an impedance analyzer; Vaz-Pires et al. (2008) characterized cuttlefish and shortfin squid  
85 stored in ice, using fish freshness meters to acquire the dielectric parameters. More recently, both  
86 Badiani et al. (2013) and Rutkayová et al., (2019) used the same freshness meters to monitor the icing  
87 of cuttlefish and common carp.

88 This paper focuses on assessing the freshness of anchovies applying image analysis, dielectric  
89 spectroscopy, and Hyper Spectral Imaging (HSI) on the fish eye. To better assess the overall quality  
90 of fish samples, mechanical properties were also monitored, performing a compression test on the  
91 fish womb. Each sampling time was measured on different fish batches to consider the whole samples  
92 variability.

93

## 94 **Material and methods**

### 95 **Anchovies' samples**

96 Anchovies (*Engraulis Encrasicolus*) were purchased soon after fishing in the Romagna region (Italy),  
97 and immediately stored in a polystyrene box covered by ice and carried in the laboratory. Fresh  
98 anchovies were stocked in a refrigerator at 0°C ( $\pm$  0.5°C). Anchovies were soon characterized: by

99 mean values and standard deviations of mass, length, and width were  $8.3 \pm 0.98$  g,  $94.7 \pm 3.4$  mm,  
100 and  $14.8 \pm 0.8$  mm, respectively. Measurements were conducted after 1, 2, 3, 4 and 7 days on different  
101 fish batches according to the assessment type. Per each storage day, different batches of fishes were  
102 investigated with the aim of considering the samples variability in the freshness assessment ability of  
103 the proposed non-destructive techniques.

104 Measurement day and final storage day were chosen on the basis of several research works evidencing  
105 the endpoint of anchovy edibility, stored at 0°C under ice, between 6 and 8 days (Massa et al., 2012;  
106 Pons-Sánchez-Cascado et al., 2006; Yapar & Yetim, 1998). All the acquisitions were conducted at  
107 room temperature.

108

#### 109 **Mechanical properties**

110 Mechanical properties were also assessed during time as contributing parameter to define the overall  
111 acceptability of fish product. Compression test was applied to anchovy womb by using a texture  
112 analyser (series Zwick-line Z 2.5; Ulm) equipped with a cylindrical probe of 2 cm of diameter and a  
113 load cell with 126 N of maximum force. Test speed was set to 1.5 mm/s. Twelve measurements were  
114 carried out for each storage time. Maximum force at yield point (hardness, N) was extrapolated.  
115 Significant differences between the means of hardness at different storage times, were evaluated by  
116 analysis of variance (ANOVA with Tukey-HSD test post hoc test, p-level > 0.05). In case of  
117 non-homogeneity of variance, evaluated by the Levene test, the non-parametric Kruskal-Wallis test  
118 (p-level < 0.05) with multiple comparison Dunn's post hoc test was applied.

119

#### 120 **Image analysis**

121 The evaluation of the visual quality of anchovy was focused on eyes and it was carried out by using  
122 an electronic eye (visual analyser VA400 IRIS Alpha M.O.S., France). For each storage time, the  
123 image acquisitions were carried out on twelve samples at 0°C.

124 The device was equipped with a resealable chamber of dimensions 420 x 560 mm, and a controlled  
125 and standardized light condition: 98 CRI (color rendering index), D65 (light of a cloudy day at 12 in  
126 the morning), 6700 °K (color temperature). In the upper part of the camera there was a CCD camera  
127 (16 million colors) for high resolution images with a built-in zoom calibrated and monitored  
128 completely automatically by the software (E-Eye software Alpha-Soft, version 14.0) capable of  
129 acquiring data, analysing images (RGB scale) and statistically processing the results. The light, inside  
130 the chamber, was emitted by two fluorescent LED channels illuminating both the upper and lower  
131 cabin sides. The back part can also be illuminated to prevent shadows from being generated on the  
132 sample. The analysis of anchovy eyes was set up with upper illumination only and resolution of  
133 1214x911 pixels. The images were acquired by placing anchovy on a removable support at 8 cm of  
134 distance to lens. The instrument performed an automatic calibration with a certified color checker,  
135 and image analysis (RGB scale or CIE  $L^*a^*b^*$ ).

136 Raw images were processed in RGB scale. Each pixel can be defined as a blend of the three primary  
137 colours of Red, Green and Blue (RGB). On a fixed scale of 4096 colors, the proportion of each colour  
138 in the analysed image is represented as a percentage. The raw images were automatically reduced by  
139 selecting the most representative colors. 108 colors were identified. ANOVA (p-level > 0.01) was  
140 applied to mean color values in order to select differences according to storage time.

141

## 142 **Dielectric properties**

143 Dielectric constant and loss factor of anchovy eyes were acquired during storage by using an open-  
144 ended coaxial probe (DAKS-3.5 probe, Speag) connected to a VNA (Vector Network Analyzer,  
145 Copper Mountains) and interfaced by USB with PC (DAK Software Installer 2.6.1.7). The  
146 instrumental chain was calibrated by using the customized calibration kit (Speag DAK-3.5/1.2  
147 Shorting Block, Metallic Strip Sets, and 0.6 lt of Tissue Simulating Liquid), accounting for open,  
148 short, and load assessment.



149 A stainless-steel support was used to fix the probe. An elevation platform was located to lead sample  
150 in contact with the coaxial probe in order to avoid possible changes due to cable movements.  
151 Dielectric properties were acquired at 23°C ( $\pm 1^\circ\text{C}$ ) in the microwave region from 250- 14000 MHz.  
152 For each storage time, seven anchovies were acquired in triplicate and evaluated in a restricted  
153 frequency range from 250 to 2400 MHz. The highest frequencies were not considered in the statistical  
154 process due to high spectral noise. Tree cluster analysis, based on Euclidean distances, was used to  
155 check similarity among mean dielectric spectra (dielectric constant and loss factor) at different storage  
156 time. Nearest neighbour aggregation rule was selected. The distance between two clusters is  
157 determined as the minimum of pairs-wise objects distances between two clusters. The clusters with  
158 minimum distance are jointly presented. Results were shown in a tree structure called dendrogram, a  
159 useful simple visualization of hierarchical structure and data clusters.

160

## 161 **Hyperspectral imaging**

162 The hyperspectral images of the anchovies were obtained through the use of a hyperspectral camera  
163 working in the spectral range from 400 to 1000 nm, for a total of 272 wavelengths (Nano Hyperspec  
164 VNIR, Headwall Photonics, Inc., Fitchburg, MA, USA). The camera works with a push broom  
165 technique, where the sample moves under the camera, which acquires the spectra of the lines of pixel  
166 one at a time. A conveyor belt was used to move the fish samples, while the correct illumination was  
167 guaranteed by two halogen lamps, mounted on the sides of the belt with an inclination of 15°.  
168 Moreover, a cardboard case was used to cover the whole system, avoiding light dispersion in the  
169 ambient. Twenty samples for each storage time were acquired at temperature of about 20° C.  
170 Reflectance spectra of white and dark reference were obtained by means of a white cardboard sheet  
171 covering the entire angle of view and placing the cover on the lens, respectively. The calibrated  
172 diffuse reflection spectrum was subsequently calculated subtracting the white and dark spectra from  
173 the raw diffuse reflectance spectrum of the fish sample.

174 A Region Of Interest (ROI) was obtained from each image, manually selecting the fish eye and the  
175 mean spectra were calculated by averaging the spectra of this region.

176 The spectral band between 400–450 nm was excluded due to low signal-to-noise-ratio produced by  
177 camera sensor, according to Wendel et al., (2018). subsequently the spectra were smoothed and pre-  
178 treated by the standard normal variate (SNV), first derivative, for scaling effects and to remove  
179 baseline offsets, and finally mean cantered.

180 Principal component analysis (PCA) was applied on the mean spectrum to visualise the data  
181 according to storage time.

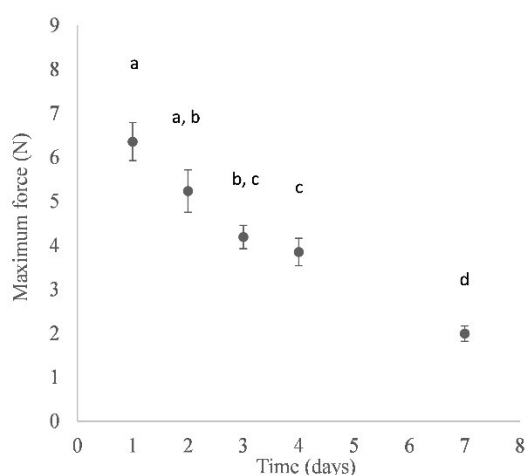
182

## 183 **Results and discussion**

184

### 185 **Mechanical properties**

186 Mean values of the maximum force after compression was shown in figure 1 according to storage  
187 time. Maximum force decrease, as a function of degradation process progress, is revealed, according  
188 to tissue softening reported by previous work (Coppes-Petricorena, 2010; Nollet & Toldrá, 2009;  
189 Yapar & Yetim, 1998).



190

191 Figure 1. Maximum force trend during storage time (mean values and related standard error) and  
192 significant differences among means (p level<0.05).

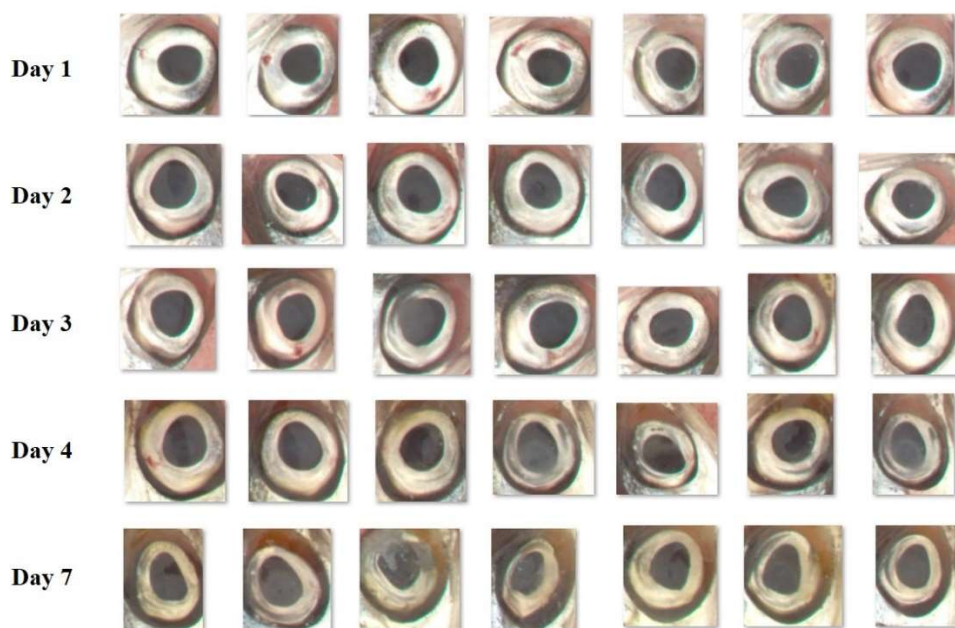
193 Significant differences between means of force for the first and the third day and between means for  
194 the last two days were detected. Good linear relation was found between mechanical properties (mean  
195 values) and storage time confirm flesh texture as good parameter for ageing detection.

196

## 197 **Image analysis**

198 Loss of surface moisture after fish death induces drying and wrinkling, and this initially occurs in the  
199 eyes (Murakoshi, Masuda, Utsumi, Tsubota, & Wada, 2013). Glossiness of fish eyes varies with time,  
200 and as a consequence wetness and brightness are lost (Murakoshi et al., 2013; Yapar & Yetim, 1998).  
201 Light refraction properties of fish eyes change due to drying. Furthermore, the material around eyes  
202 is transferred into eyes increasing fluid concentration (Gokoglu & Yerlikaya, 2004). Concerning these  
203 mechanisms, glossiness, refractive index, transparency have been since a long time used for fish  
204 freshness estimation (Gokoglu & Yerlikaya, 2004; Murakoshi et al., 2013; Yapar & Yetim, 1998).  
205 Large consents have been observed since visual perception is strictly related to consumer acceptance.  
206 In the light of previous works, traditional approach, such as image analysis and mechanical properties  
207 were applied to understand ageing behaviour, while new application like dielectric properties and  
208 hyperspectral imaging were applied to develop new methods with potential practical application.

209 Images of anchovy eyes during storage were captured as shown in figure 2.



210

Figure 2. Examples of eyes modification during storage.

A difference, passing from day 1 to 7 day, can be observed from figure 2, confirming the validity of image method. As expected, colour of anchovy eyes changes as a consequence of ageing. ANOVA was applied to select colors significant modification as a function of storage, and it results 60 significative colors among 108. Some examples were shown in figure 3.

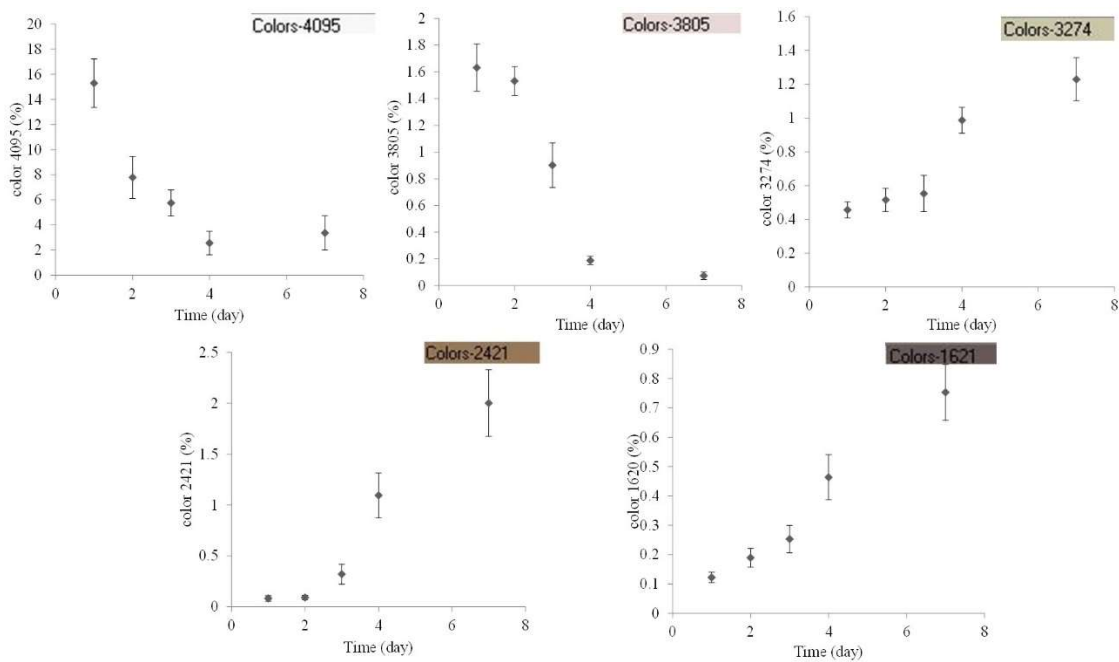
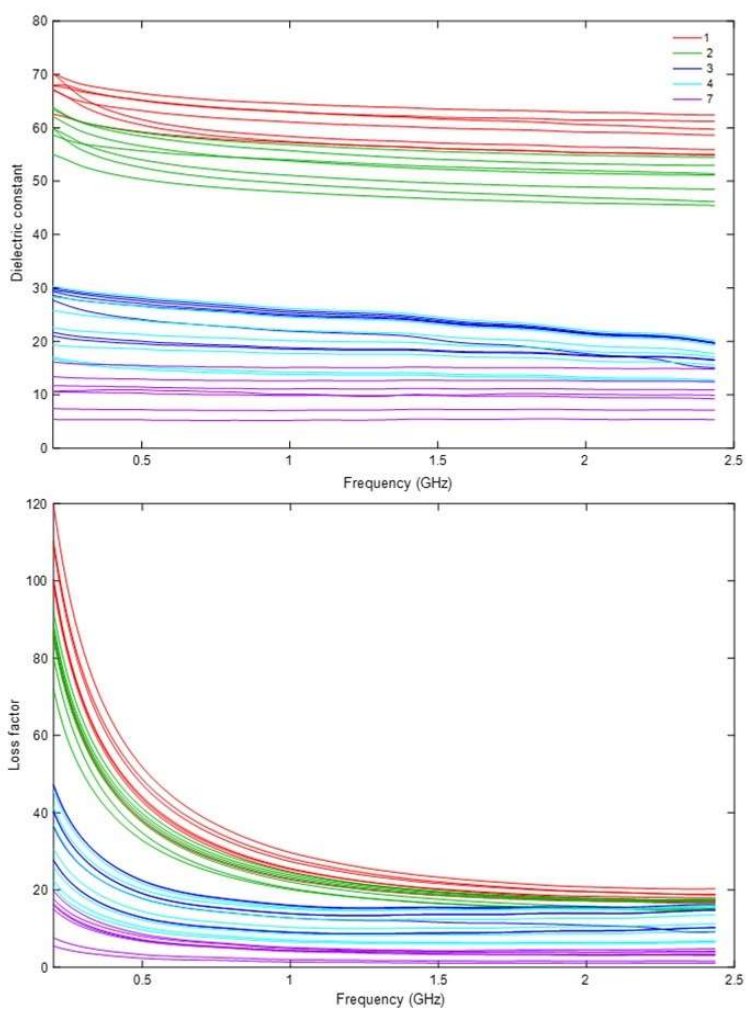


Figure 3. Mean values and standard errors trend of colors during storage time.

Color percentages (mean values) of light colours such as 4095 and 3805 decrease, while an opposite trend was observed for dark colours so 3274, 2421 and 1621 increase during storage time. The two dark colors increase is probably due to post mortem mechanism, while light colours seems marker of freshness, as they are lost during time, as previously reported for glossiness, refractive index, and transparency (Gokoglu & Yerlikaya, 2004; Murakoshi et al., 2013; Negi et al., 2019).

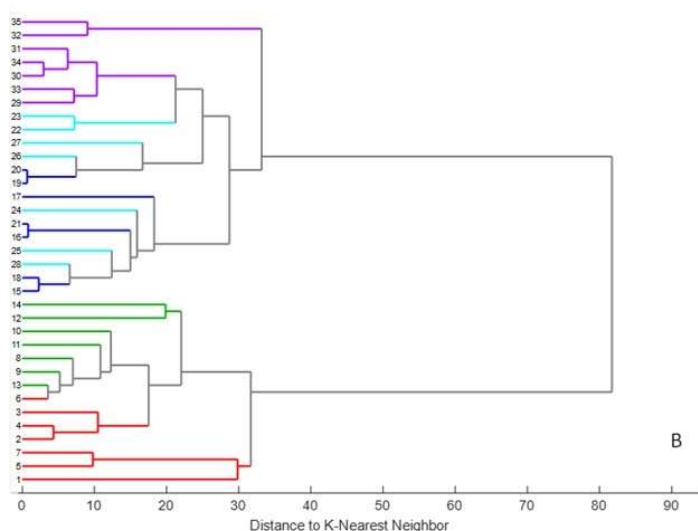
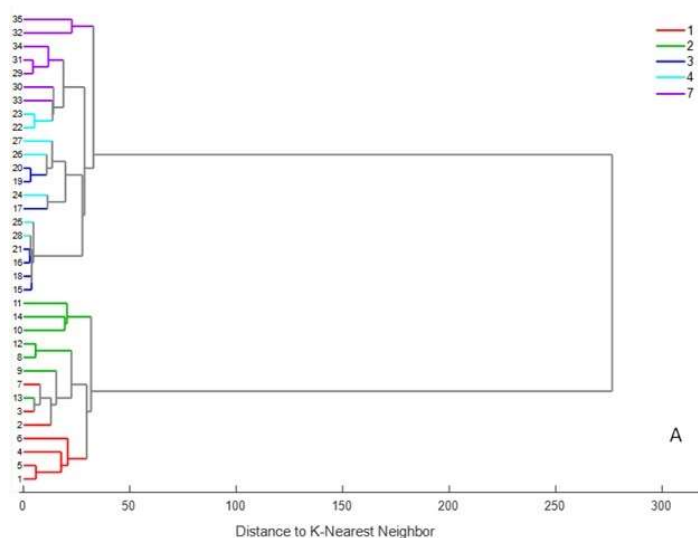
## Dielectric properties

227 For a long time, dielectric properties of foodstuff were investigated as a non-destructive and rapid  
228 method for quality assessment (Ryynänen, 1995). Considering freshness of fish electric meter were  
229 developed with different electrode arrangement measuring conductance and capacitance of fish  
230 muscle (Olafsdottir et al., 2004). Accordingly, dielectric properties were still used also for the  
231 suitability for sensor development. Fish eyes dielectric properties were not previously considered by  
232 literature, even if it seems the best way to determine freshness as already mentioned.  
233 The dielectric constant and loss factor of anchovy eyes for all the considered storage time were shown  
234 in figure 4.



235  
236 Figure 4. Dielectric constant ( $\epsilon'$ ) and loss factor ( $\epsilon''$ ) measured during storage time, legend reports  
237 storage days.

239 As mentioned before, fish eye characteristics are recognized as freshness index. Both dielectric  
240 parameters present high spectra variability as a function of eyes freshness loss. As expected,  
241 increasing storage time corresponds with a decrease in dielectric constant and loss factor of fish eyes.  
242 This trend can be explained by a reduction of surface moisture of eyes, and characteristic  
243 modifications due to post-mortem mechanisms. Reduction of water is traduced in reduction of dipoles  
244 decreasing polarization and ion conductivity involving in complex permittivity adaptation (Traffano-  
245 Schiffo, Castro-Giraldez, Colom, Talens, & Fito, 2021). Furthermore, the low frequency range  
246 observed and a slight decrease of dielectric constant as a function of frequency highlight the existence  
247 of  $\gamma$  dispersion related to relaxation of water of hydration (Gabriel, Sheppard, & Grant, 1983).  
248 Accordingly, the dielectric measurement confirming a modification in the hydration state of the  
249 substrate was analysed. The cluster analysis results carried out for dielectric constant and loss factor  
250 spectra, are shown in figure 5.



251

252 Figure 5. Dendrogram from dielectric constant ( $\epsilon'$ , A) loss factor ( $\epsilon''$ , B) spectra discriminating  
 253 anchovies as a function of time (days, from 1 to 7).

254

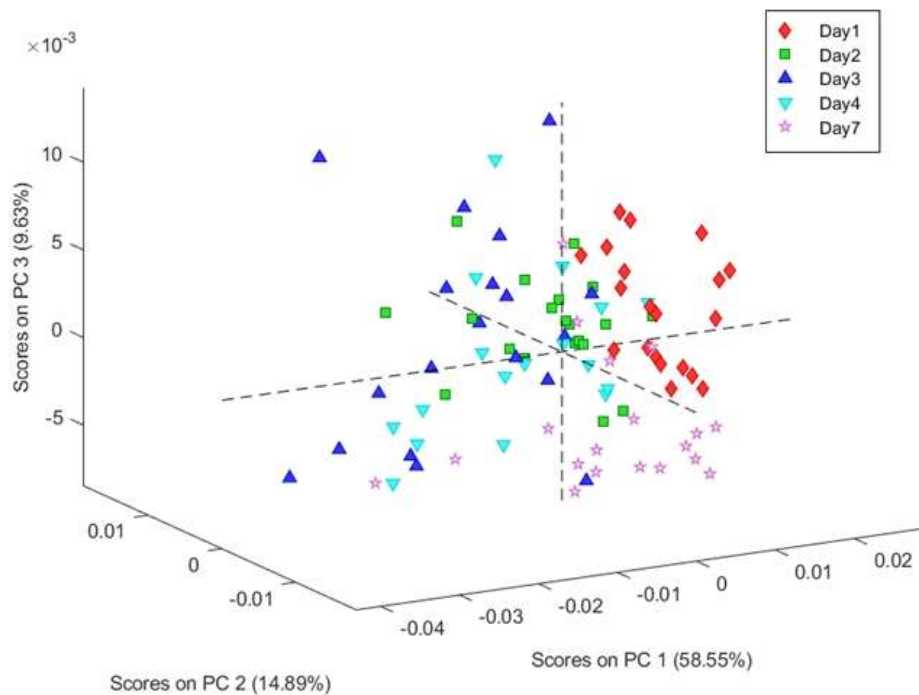
255 Similar samples involved in cluster and progress are jointed together for similarity based on distance  
 256 rules. Cluster analysis showed a good discrimination according to different days for dielectric  
 257 constant and loss factor. Days 3 and 4 are not quite different involving in two mixed clusters. Clear  
 258 clusters are visible from days 1 and 2 to 3, 4 and 7 days of storage.

259 Not a clear difference emerged in terms of spectral information contained in the dielectric constant  
 260 and loss factor.

261

262   **Hyperspectral imaging**

263   Concerning the hyperspectral image, the 3D score plot of the PCA is reported in figure 6.



264

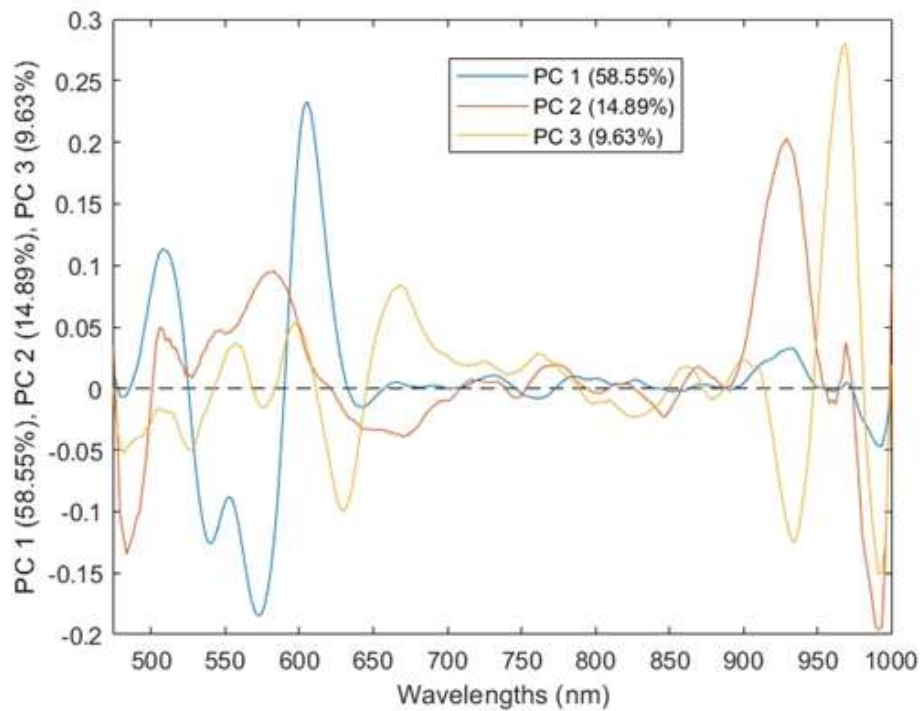
265   Figure 6. Score plot of PCA model created with hyperspectral data, showing the first three PCs.

266

267   According to storage time, good separation between the samples, according to the storage time, was  
268   achieved. Especially along the 1<sup>st</sup> PC (day1 vs day2 vs day3) and the 3<sup>rd</sup> PC (days1, 2, and3 vs day7).

269   The loading plot in figure 7 can help to understand which physical changes are described by the PCs,  
270   showing how much each measured wavelength contributes to the variance explained by these new  
271   directions.





272

273 Figure 7. Loading plot of the PCA model created with hyperspectral data, showing the first three  
274 PCs.

275

276 It apparent that all the PCs have high loading values in the range of the visible (500-700 nm),  
277 especially the first PC, that alone explain the 58.55% of the data variance, pointing to changes in the  
278 sample colors during the decaying process. The second and third PC instead present maximum  
279 loadings values in the range of 900-1000 nm, in proximity to one of the absorption band of the water  
280 (970 nm): as already described for the dielectric spectra, the moisture and water content of anchovies  
281 changes greatly during the 7 days.

282

## 283 Conclusions

284 Freshness of anchovies was assessed by considering different batches of fish for each sampling time.  
285 The obtained results suggest that freshness index of anchovy measured by optical properties,  
286 dielectric spectroscopy, and hyperspectral technique could be proposed as a reliable alternative to the  
287 traditional sensory analysis. Sensory analysis is time-consuming and subjective while instrumental  
288 methods are able to be sensitive, non-destructive and with possibly implemented as automatic control.

289 Image analysis and texture measurement confirmed an excellent ability to estimates aging, as  
290 significant trend of force and colors reported. Dielectric spectroscopy and hyperspectral images  
291 were proposed as alternative techniques for rapid and non-destructive features. Both techniques result  
292 able to group samples according to storage days arise promising for practical application, also  
293 considering that analysed fish were different for each storage time describing the real samples  
294 variability. The present results are potentially useful not only for determining the mechanism of fish  
295 freshness, but also towards developing new techniques for the non-destructive evaluation of the  
296 freshness of fish.

297 Hyperspectral imaging results could be used to further study in order to select some wavelength of  
298 interest and developed a rapid, contactless, and more affordable system based on multispectral camera  
299 for fish freshness assessment.

300 Dielectric technique seems the promising technique revealing a good ability to characterize eyes  
301 dehydration during ageing and suitability for the development of a compact instrument, in a restricted  
302 frequency range, equipped with customized probe, acquisition and elaboration system for rapid  
303 decisions.

304

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