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RADIO-FREQUENCY AND OPTICAL TECHNIQUES FOR ANCHOVY FRESHNESS EVALUATION

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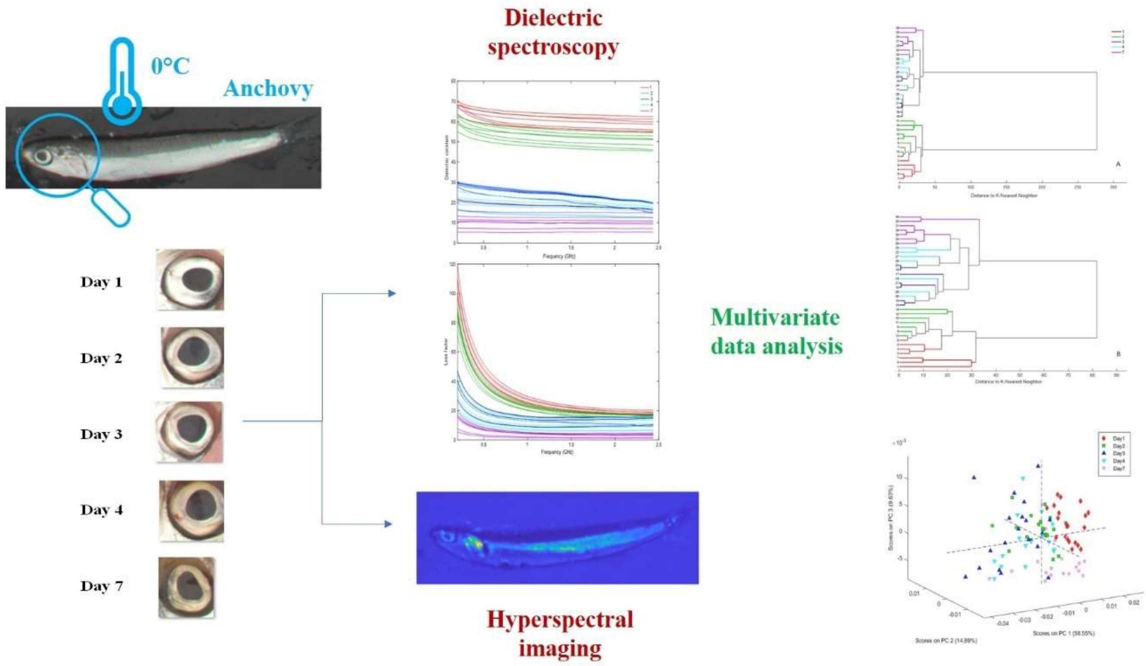

Abstract

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

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

Introduction

Mediterranean anchovy (*Engraulis encrasicholus*) plays a key role both in the human diet, thanks to the high presence of protein and n-3 fatty acids in its meat (Gencbay & Turhan, 2016), and in the economy of the Mediterranean countries. An average of 270,000 tons are landed every year in the whole Mediterranean basin (FAO, 2018). The most important aspect to consider and monitor, not only in anchovies, but in the whole fish industry and commercialization, is freshness: after a brief period of time, dead fishes show an increase in the growth and activity of microorganism and in the oxidation of lipids (Erkan, Özden, Alakavuk, Yildirim, & Inuğur, 2006). These two processes cause a fast deterioration of critical quality parameters for the consumers, like appearance, odor and taste (Kyrana & Lougovois, 2002; Olafsdottir, Jonsdottir, Lauzon, Luten, & Kristbergsson, 2005). The speed and severity of these changes are highly variable, depending on several parameters like species, fat content, storage conditions and temperature (Alasalvar, Taylor, Öksüz, Shahidi, & Alexis, 2002; Ashie, Smith, & Simpson, 1996; Olafsdottir et al., 2004). Moreover, the refrigeration process does not stop the whole microbial activity, thanks to the presence of psychotropic bacteria (Pedrosa-Menabrito & Regenstein, 1988). The traditional method to assess fish freshness is the sensory inspection, used by the European Union since the ’70 (CEE, 1976). Nowadays, the most common 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 asses 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; Velioǐlu, 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 monitoring. Also the study of more straighforward RBG images (usually focusing on eyes and gills) represents an active field, as evidenced by recent works on common carp and goldfish (Bachrun Alim, Suhaeli Fahmi, Purnamayati, & Agustini, 2020; Negi, Yadav, Rawat, & Singh, 2019) and rainbow trout (Mohammadi Lalabadi, Sadeghi, & Mireei, 2020).

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

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

Material and methods

Anchovies' samples

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

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

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

Mechanical properties

Mechanical properties were also assessed during time as contributing parameter to define the overall acceptability of fish product. Compression test was applied to anchovy womb by using a texture analyser (series Zwick-line Z 2.5; Ulm) equipped with a cylindrical probe of 2 cm of diameter and a load cell with 126 N of maximum force. Test speed was set to 1.5 mm/s. Twelve measurements were carried out for each storage time. Maximum force at yield point (hardness, N) was extrapolated.

Significant differences between the means of hardness at different storage times, were evaluated by analysis of variance (ANOVA with Tukey-HSD test post hoc test, p-level > 0.05). In case of non-homogeneity of variance, evaluated by the Levene test, the non-parametric Kruskal-Wallis test (p-level < 0.05) with multiple comparison Dunn's post hoc test was applied.

Image analysis

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

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

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

Dielectric properties

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

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

Hyperspectral imaging

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

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

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

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

Results and discussion

Mechanical properties

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

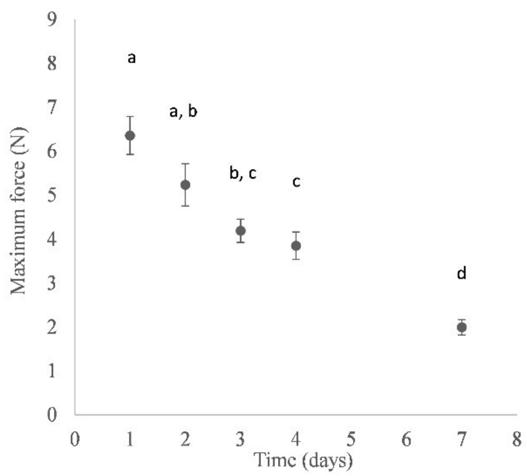

Figure 1. Maximum force trend during storage time (mean values and related standard error) and significant differences among means (p level<0.05).

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

Image analysis

Loss of surface moisture after fish death induces drying and wrinkling, and this initially occurs in the eyes (Murakoshi, Masuda, Utsumi, Tsubota, & Wada, 2013). Glossiness of fish eyes varies with time, and as a consequence wetness and brightness are lost (Murakoshi et al., 2013; Yapar & Yetim, 1998). Light refraction properties of fish eyes change due to drying. Furthermore, the material around eyes is transferred into eyes increasing fluid concentration (Gokoglu & Yerlikaya, 2004). Concerning these mechanisms, glossiness, refractive index, transparency have been since a long time used for fish freshness estimation (Gokoglu & Yerlikaya, 2004; Murakoshi et al., 2013; Yapar & Yetim, 1998). Large consents have been observed since visual perception is strictly related to consumer acceptance. In the light of previous works, traditional approach, such as image analysis and mechanical properties were applied to understand ageing behaviour, while new application like dielectric properties and hyperspectral imaging were applied to develop new methods with potential practical application. Images of anchovy eyes during storage were captured as shown in figure 2.

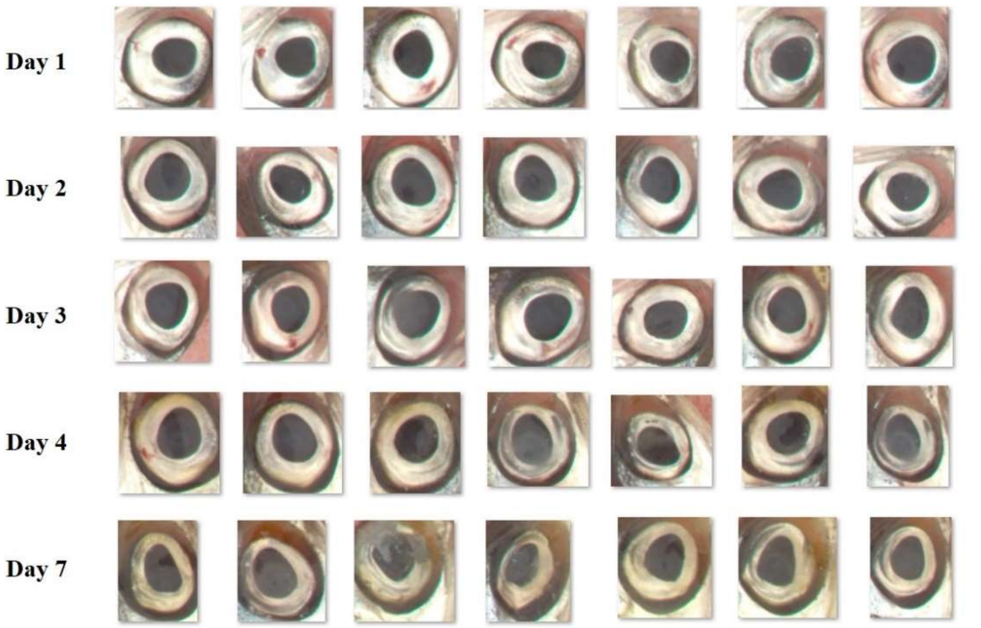

Figure 2. Examples of eyes modification during storage.

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.

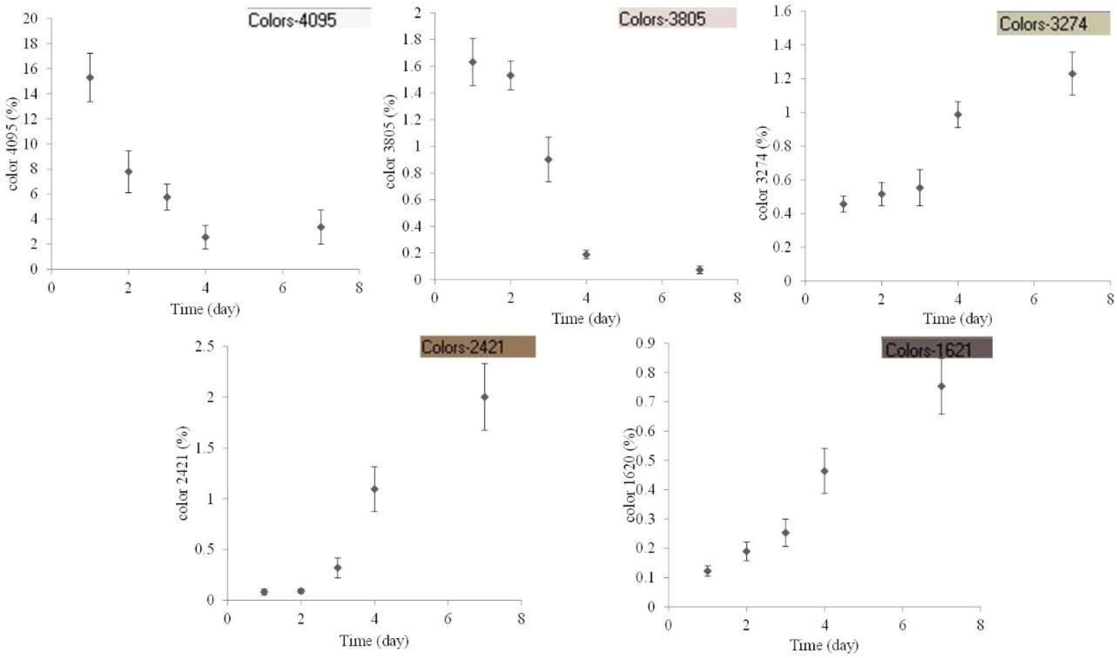

Figure 3. Mean values and standard errors trend of colors during storage time.

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

For a long time, dielectric properties of foodstuff were investigated as a non-destructive and rapid method for quality assessment (Ryynänen, 1995). Considering freshness of fish electric meter were developed with different electrode arrangement measuring conductance and capacitance of fish muscle (Olafsdottir et al., 2004). Accordingly, dielectric properties were still used also for the suitability for sensor development. Fish eyes dielectric properties were not previously considered by literature, even if it seems the best way to determine freshness as already mentioned.

The dielectric constant and loss factor of anchovy eyes for all the considered storage time were shown in figure 4.

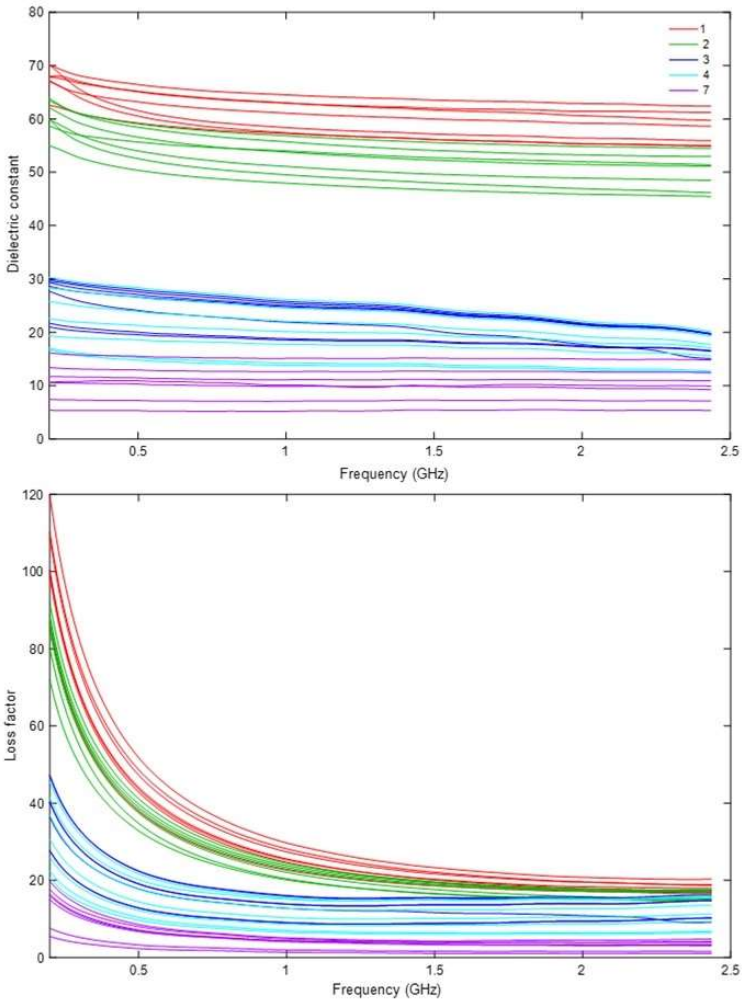

Figure 4. Dielectric constant (ε’) and loss factor (ε’’) measured during storage time, legend reports storage days.

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

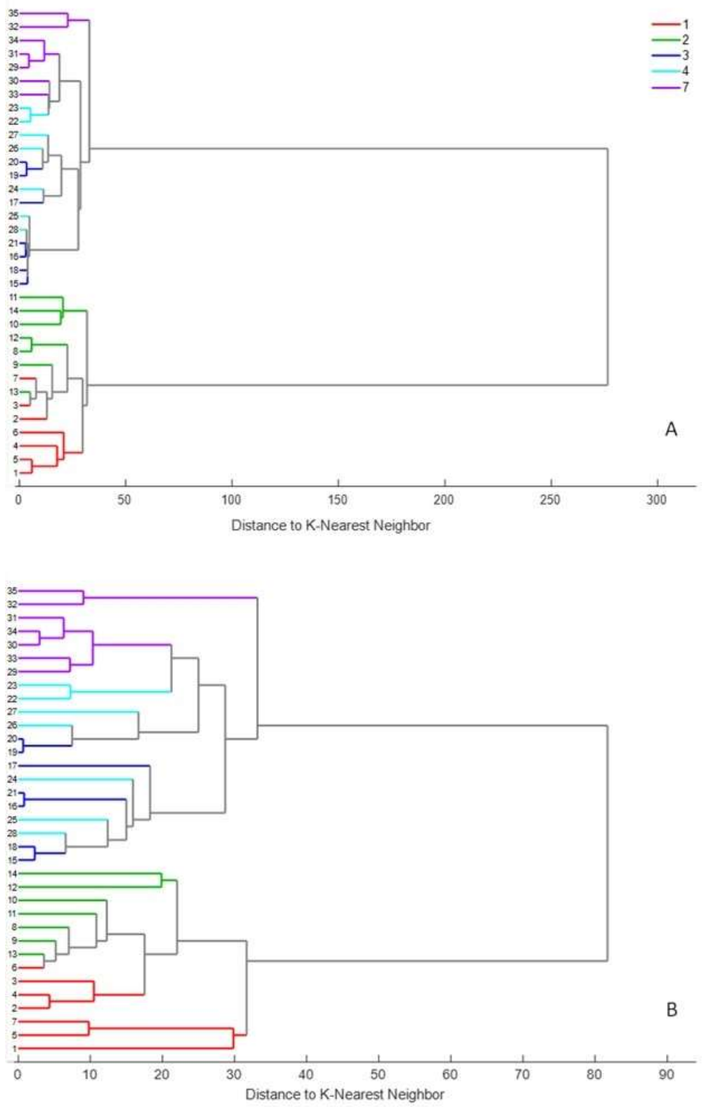

Figure 5. Dendrogram from dielectric constant (ε', A) loss factor (ε'', B) spectra discriminating anchovies as a function of time (days, from 1 to 7).

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

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

Hyperspectral imaging

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

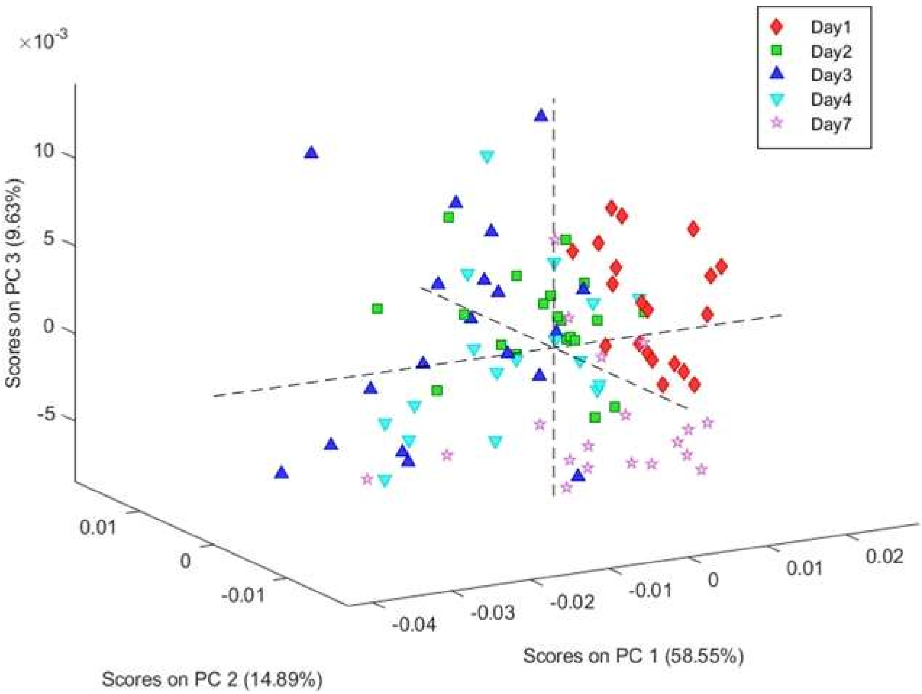

Figure 6. Score plot of PCA model created with hyperspectral data, showing the first three PCs.

According to storage time, good separation between the samples, according to the storage time, was achieved. Especially along the 1st PC (day1 vs day2 vs day3) and the 3rd PC (days1, 2, and3 vs day7). The loading plot in figure 7 can help to understand which physical changes are described by the PCs, showing how much each measured wavelength contributes to the variance explained by these new directions.

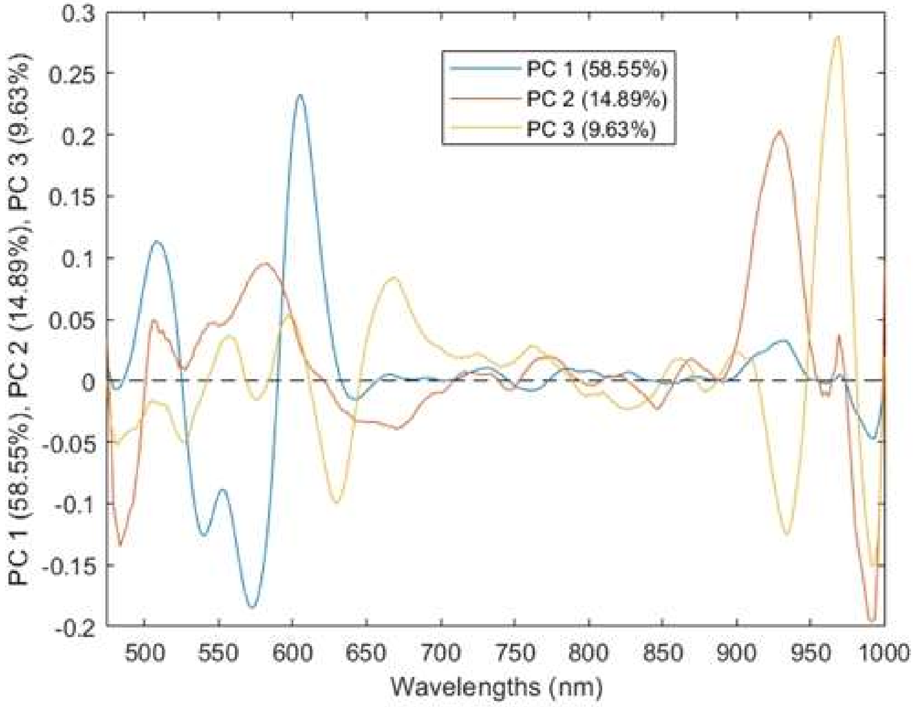

Figure 7. Loading plot of the PCA model created with hyperspectral data, showing the first three PCs.

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

Conclusions

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

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

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

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

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