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9	Corresponding Author	Organization CRA-ING Agricultural Engineering Research Unit of the Agriculture Research Council
10	Division	
11	Address	Via della Pascolare, 16, Monterotondo Scalo, Rome 00015, Italy
12	e-mail	corrado_costa@libero.it
13	Family Name	Antonucci
14	Particle	
15	Given Name	Francesca
16	Suffix	
17	Author	Organization CRA-ING Agricultural Engineering Research Unit of the Agriculture Research Council
18	Division	
19	Address	Via della Pascolare, 16, Monterotondo Scalo, Rome 00015, Italy
20	e-mail	
21	Family Name	Menesatti
22	Particle	
23	Given Name	Paolo
24	Author	Suffix
25	Organization	CRA-ING Agricultural Engineering Research Unit of the Agriculture Research Council
26	Division	

27	Address	Via della Pascolare, 16, Monterotondo Scalo, Rome 00015, Italy
28	e-mail	
29	Family Name	Pallottino
30	Particle	
31	Given Name	Federico
32	Suffix	
33	Author	Organization CRA-ING Agricultural Engineering Research Unit of the Agriculture Research Council
34	Division	
35	Address	Via della Pascolare, 16, Monterotondo Scalo, Rome 00015, Italy
36	e-mail	
37	Family Name	Boglione
38	Particle	
39	Given Name	Clara
40	Suffix	
41	Author	Organization University of Rome "Tor Vergata"
42	Division	Laboratory of Experimental Ecology and Aquaculture, Department of Biology
43	Address	Via della Ricerca Scientifica, Rome 00133, Italy
44	e-mail	
45	Family Name	Cataudella
46	Particle	
47	Given Name	Stefano
48	Suffix	
49	Author	Organization University of Rome "Tor Vergata"
50	Division	Laboratory of Experimental Ecology and Aquaculture, Department of Biology
51	Address	Via della Ricerca Scientifica, Rome 00133, Italy
52	e-mail	
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56	Abstract	Freshness represents a pivotal aspect in fish product for both security and quality. Its evaluation still represents the key factor driving the consumer' choices. Fish appearance is affected by many different factors that demand the contribution of different disciplines to be understood: from the physical and optical properties to the slaughtering and post-slaughtering conditions. An innovative preservation system is represented by the

Passive Refrigeration PRS™ developed for the preservation and transport of perishable food products. Scientific methods for product freshness evaluation may be conveniently divided into two categories: sensorial and instrumental. In this study, an instrumental method of colour calibration and discrimination is proposed at pilot scale for automatic evaluation of gilthead seabream (*Sparus aurata*) freshness. We propose a non-destructive method based on the colorimetric imaging of the whole external body of seabreams to evaluate through multivariate partial least squares which approach the differences in the freshness preservation under four refrigeration modalities. The matrix of the independent variables is represented by RGB values for each pixel belonging to an extracted region of interest (129,633 values). The dependent variable is composed by two dummy variable corresponding to fresh (T_0) or non-fresh (T_2) individuals. T_1 individuals were used as external test. The results quantified significant colorimetric differences between fresh and non-fresh fish. All fish used to create the model (T_0 and T_2) were correctly classified as fresh or non-fresh, while external test individuals (T_1) were all classified as fresh. The proposed imaging method merges different image analysis techniques: (a) colorimetric calibration, (b) morphometric superimposition and (c) partial least square discriminant analysis modelling. This innovative and non-destructive approach allows the automatic assessment of fish freshness.

57 Keywords Fish freshness assessment - Gilthead seabream - Colorimetric calibration -
separated by ' - ' PLS - Warping - Passive refrigeration system

58 Foot note
information

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COMMUNICATION

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An Advanced Colour Calibration Method for Fish Freshness Assessment: a Comparison Between Standard and Passive Refrigeration Modalities

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Corrado Costa · Francesca Antonucci ·
Paolo Menesatti · Federico Pallottino · Clara Boglione ·
Stefano Cataudella

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Abstract Freshness represents a pivotal aspect in fish product for both security and quality. Its evaluation still represents the key factor driving the consumer' choices. Fish appearance is affected by many different factors that demand the contribution of different disciplines to be understood: from the physical and optical properties to the slaughtering and post-slaughtering conditions. An innovative preservation system is represented by the Passive Refrigeration PRS™ developed for the preservation and transport of perishable food products. Scientific methods for product freshness evaluation may be conveniently divided into two categories: sensorial and instrumental. In this study, an instrumental method of colour calibration and discrimination is proposed at pilot scale for automatic evaluation of gilthead seabream (*Sparus aurata*) freshness. We propose a non-destructive method based on the colorimetric imaging of the whole external body of seabreams to evaluate through multivariate partial least squares which approach the differences in the freshness preservation under four refrigeration modalities. The matrix of the independent variables is represented by RGB values for each pixel belonging to an extracted region of interest (129,633 values). The dependent variable is composed by two dummy variable corresponding to fresh (T_0) or non-fresh (T_2) individuals. T_1

individuals were used as external test. The results quantified significant colorimetric differences between fresh and non-fresh fish. All fish used to create the model (T_0 and T_2) were correctly classified as fresh or non-fresh, while external test individuals (T_1) were all classified as fresh. The proposed imaging method merges different image analysis techniques: (a) colorimetric calibration, (b) morphometric superimposition and (c) partial least square discriminant analysis modeling. This innovative and non-destructive approach allows the automatic assessment of fish freshness.

Keywords Fish freshness assessment · Gilthead seabream · Colorimetric calibration · PLS · Warping · Passive refrigeration system

Introduction 49

Appearance is used throughout the production, storage, marketing and utilisation chain as the primary means of judging the quality of individual units of product. Fish appearance can be due to many different factors such as optical properties, physical form and health status, chemical composition and microbial load, method of slaughtering and preservation and the environmental conditions in which it has lived. The most important conservation factor is to chill fish with ice to about 0 °C to increase the thermal stability but this requires a huge amount of ice (1:1, Jeyasekaran et al. 2004) and produce drip loss, textural toughness, nutrient loss and decreases in protein extractability (Putro 1989). Lately, an innovative preservation system (Passive Refrigeration PRS™—NOMOS S.p.a. Olgiate Molgora, Italy) was developed for the preservation and transport of perishable products. The system guarantees perfect shelf life preservation through the maintenance of

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C. Costa (✉) · F. Antonucci · P. Menesatti · F. Pallottino
CRA-ING Agricultural Engineering Research
Unit of the Agriculture Research Council,
Via della Pascolare, 16, Monterotondo Scalo,
00015 Rome, Italy
e-mail: corrado_costa@libero.it

C. Boglione · S. Cataudella
Laboratory of Experimental Ecology and Aquaculture,
Department of Biology, University of Rome "Tor Vergata",
Via della Ricerca Scientifica,
00133 Rome, Italy

66 optimal temperature and relative humidity, without the use of
67 power during operation.

68 As reported by Olafsdottir et al. (2004), fish freshness
69 evaluation carried out by physical techniques is generally more
70 rapid than with chemical ones, indeed optical and electrical
71 measurements are almost instantaneous. Since the consumer is
72 the ultimate judge of quality, most instrumental methods must
73 be correlated with sensorial measures related to the sight, the
74 touch or the odour perception (Menesatti et al. 2010; Quevedo
75 et al. 2010). For whole fish the EU quality grading scheme
76 (Howgate et al. 1992) is used as required by EU regulation
77 (European Community 1996), but some initiatives have been
78 taken to implement a new sensory method named Quality
79 Index Method (QIM) to standardise sensory assessment for
80 each species (Martinsdottir et al. 2004; Olafsdottir et al. 2004).
81 Sensory attributes influencing the freshness and quality of fish
82 related to appearance, texture, smell, colour, defects and hand-
83 dling were all considered very important (Quevedo et al.
84 2010).

85 Generally, species-specific colour is a critical sensory
86 characteristic of fish quality as it is used by consumers as
87 an indicator of the perceived quality and freshness. All sets
88 of colour values show a fairly good linear relationship with
89 both the QIM values and the values for appearance of skin
90 (Olafsdottir et al. 2004). The functioning of modern color-
91 imeters is comparable to the principle of colour perception
92 used by the human eye (Li-Tsang et al. 2003).

93 In this scenario, this study aimed to test the ability of a
94 novel, automated and non-destructive methodology of assess-
95 ing the freshness of whole fishes, based on external colour
96 appearance of samples preserved with a conventional system
97 and an innovative passive refrigerator. Digital images of
98 whole gilthead seabream (*Sparus aurata* Linnaeus, 1758)
99 were taken soon after harvest, after four different refrigeration
100 modalities and following three different periods of preserva-
101 tion were calibrated, with respect to a standard colour chart,
102 with a Partial Least Squares (PLS) approach.

103 **Materials and Methods**

104 **Samples**

105 Thirty gilthead seabreams reared at the commercial farm
106 Civitaltica s.r.l. (Civitavecchia, Italy) were used for the
107 experiments. The fishes were split in four groups, following
108 four refrigeration modalities: (a) stored according to tradi-
109 tional market techniques, in a polystyrene tray covered with
110 a plastic seafood film (as commonly practiced in Italy), with
111 crushed ice placed above the film and placed into an indus-
112 trial refrigerator (0.6 m³) at 2 °C for 5 days (Frg, eight fish);
113 (b) stored in a polystyrene tray covered with a plastic sea-
114 food film with crushed ice placed above the film and placed

outside for 1 day (7–12 °C), in order to simulate a market 115
exposition, and then placed into an industrial refrigerator 116
laying on the right side (0.6 m³) at 2 °C for 5 days (Out, 117
seven fish); (c) stored in a polystyrene tray covered with a 118
plastic seafood film, without ice, and placed into a PRSTM 119
Passive Refrigeration System Thermopallet EI (1.93 m³; 120
1.21×0.81×1.95 m height) at 2 °C for 5 days (Prs, eight 121
fish); (d) stored in a polystyrene tray covered with a plastic 122
seafood film, displayed into an industrial freezer at -10 °C 123
for 3 days and then thawed during placement into an indus- 124
trial refrigerator (0.6 m³) at 2 °C for 2 days (Frz, seven fish). 125
Each fish group was analysed after 4 h *post-mortem* (T₀) and 126
after 2 days (T₁), except for Frz, and after 5 days (T₂). The 127
fish used were sampled within the commercial size of gilt- 128
head seabreams (mean body weight=342.8±32.3 g). 129

130 Thermo-hygrometric data inside the PRS and the indus-
131 trial refrigerator were acquired through automated acquisi-
132 tion instruments (every 5 min throughout the testing period),
133 consisting of air temperature and relative humidity (RH)
134 sensors integrated with a datalogger (H2 Testo AG. 135
Lenzkirch-DE: precision 0.5 °C for temperature e, 1% for
136 relative humidity). The temperature inside the conventional
137 industrial refrigerator was measured also in contact with ice.
138 The weight loss of samples was measured for each refriger-
139 ation modality and conservation time above mentioned.

140 **PRSTM Passive Refrigeration System**

141 The system is composed of two units: (a) the container,
142 internally hosting the products and built with walls filled
143 with eutectic liquid solution of water and salt to obtain a
144 specific ice fusion point temperature, and (b) the refrigera-
145 tion unit, filled with ethylene glycol that cools down and
146 sends back to the first unit through a closed circuit. Once the
147 product unit is fully charged and the walls of the system are
148 frozen, it can hold the temperature for several days or,
149 inversely, it can be used attached to the charging unit, after
150 setting the most appropriate temperature. In both cases, the
151 products are stored with a high percentage of humidity and a
152 very low ΔT .

153 **Digital Image Acquisition and Processing**

154 In order to measure the colour pattern, the camera was
155 mounted on a tripod and images of single fish were acquired.
156 For the acquisition, the samples were taken out from each
157 refrigeration system for about 2 min. A high-resolution Nikon
158 Coolpix P6000 camera (13.5 real MP) was used to acquire
159 TIFF 8-bit images. Manual white balance control, exposure
160 and metering methods were enabled. ISO sensibility was set to
161 100 to avoid any noise appearance. Colour calibration and
162 validation were carried out using a GretagMacbeth Color-
163 Checker 24 colour patch, as reference standard. Samples were
164

164 illuminated with four photographic low-consumption gas
 165 lamps with a power of 60 W, producing a light corresponding
 166 to 270 W of the traditional bulbs. Such lamps present a
 167 nominal illumination power of 3,800 lm, paired with a light
 168 temperature of 5,000 K (daylight) and an electronic converter
 169 that avoids the flickering effect.

170 Matlab (rel. 7.1, PLSToolbox Eigenvector rel. 4.0) was
 171 used to perform the image calibration based on PLS calibration
 172 (Costa et al. 2009a). RGB declared values of the Color-
 173 Checker (24 patch) were used as *y*-block. The *x*-block was
 174 represented by the mean RGB value of the same 24 patch.

175 Colorimetric Warping Analysis

176 After colour calibration, a total number of 18 landmarks were
 177 digitised (Fig. 1a) on the left side of each fish image, in order
 178 to allow the comparison of the entire body fish area. The first
 179 13 landmarks were used to determine the region of interest
 180 (ROI) to be compared among samples. Following the land-
 181 mark configuration, the image RGB matrices were warped
 182 through a geometric morphometric procedure (Costa et al.
 183 2009b; Menesatti et al. 2010). In this way, each pixel inside
 184 each ROI could be compared with the one in the same position
 185 of the other samples. For each individual, the three RGB
 186 values of the 43,211 pixels composing the ROI were decom-
 187 posed in a single row (129,633 values).

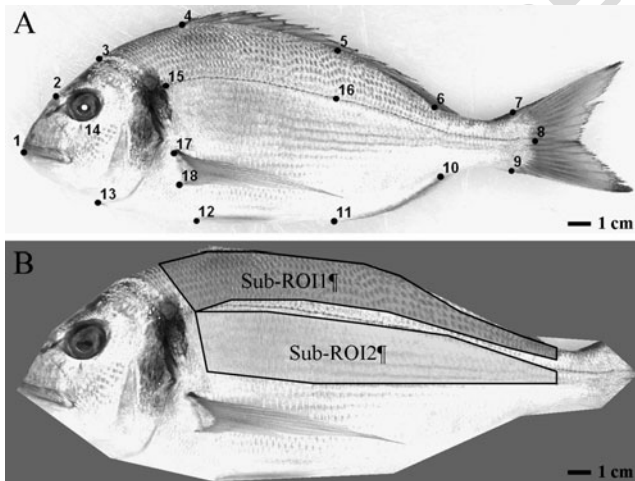


Fig. 1 **a** Landmarks used for the warping procedure. Description: 1 snout tip; 2 position of the gold stripe on the profile; 3 curvilinear projection of the opercular plate on the profile; 4 insertion of anteriormost dorsal spine; 5 insertion of anteriormost soft dorsal ray; 6 insertion of the posteriormost soft ray; 7 and 9 dorsal and ventral insertion of the caudal fin; 8 posterior most caudal peduncle extremity; 10 and 11 posterior and anterior insertion of the anal fin; 12 insertion of the pelvic fin; 13 ventro-lateral insertion of the opercular plate; 14 centre of the eye; 15 begin of trunk lateral line; 16 vertical projection of the anteriormost soft dorsal ray on the lateral line; 17 and 18 upper (dorsal) and lower (ventral) insertion of the pectoral fin. **b** The two Sub-ROIs (region of interest; *Sub-ROI1*, *Sub-ROI2*) identified by the major pixel's contribution to the PLSDA classification

Statistical Analyses

The matrix (60×129,633) representing the RGB colour values inside the ROI of each seabream at T_0 and T_2 was analysed with a Partial Least Square Discriminant Analysis (PLSDA; Sabatier et al. 2003; Costa et al. 2010). PLS is a soft modelling method for constructing predictive models with many and highly collinear factors. The technique looks for correlations among the 129,633 RGB values of each pixel (*x*-block); the *y*-block was composed by two dummy variables correspondent to fresh (T_0) or non-fresh (T_2) individuals. The *x*-block was pre-processed with an 'autoscale' procedure. The load of each pixel (*x*-block), in each latent vector (LV), was extracted (Costa et al. 2009b) in order to determine the pixel's contribution to the classification (fresh vs non-fresh). Thirty individuals at T_1 were used as external test.

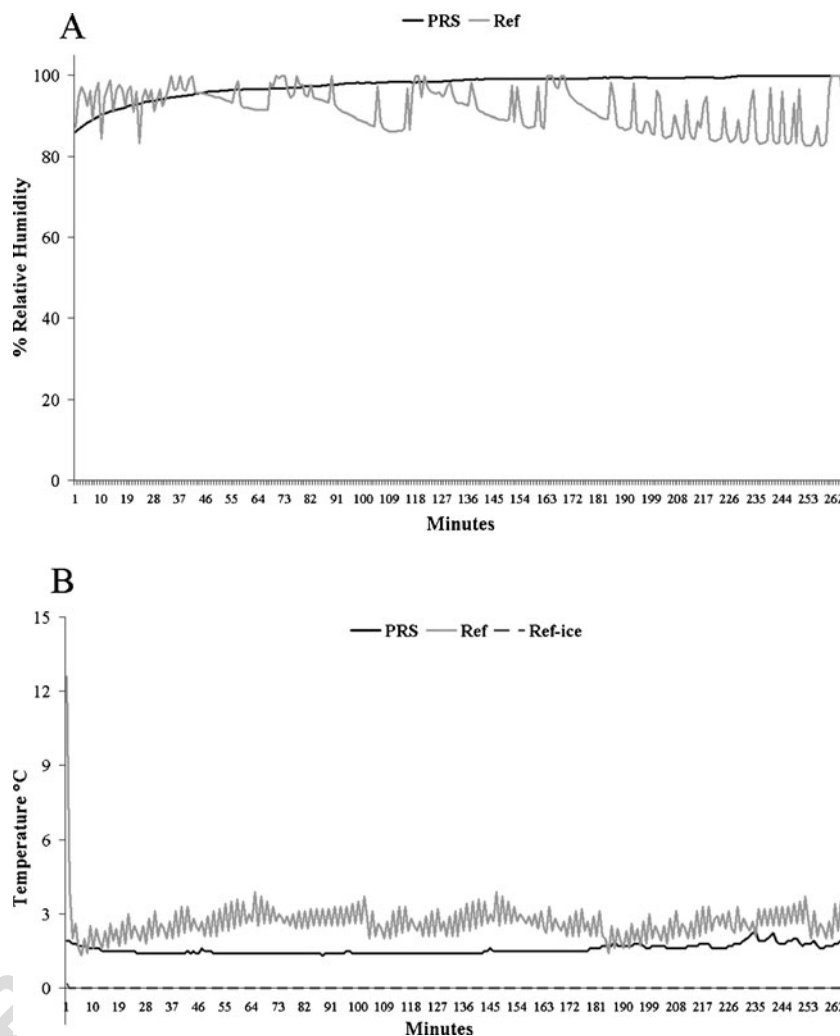
Basing on the pixel's contribution to the classification of PLSDA, two ROIs were identified (*Sub-ROI1*, *Sub-ROI2*), one below the dorsal fins and the other below the lateral line (Fig. 1b). The RGB mean values on these ROIs were extracted to statistically test the significance of differences with repeated measures MANOVA. Such comparison was carried out within *Sub-ROI1* values and *Sub-ROI2* values (not between them), respectively, at T_0 and T_2 . A dendrogram based on the mean Euclidean distances, between the different refrigeration modalities and conservation times, based on the three RGB values decomposed in a single row (129,633 values), was built.

Results and Discussion

The PRSTM resulted as the best of the two tested refrigerator systems: as show in Fig. 2, the RH% and temperature trend lines of the two systems show totally different trends. The RH% industrial refrigerator oscillates between 83% and 100%, repeatedly during all the conservation time. Such a trend affects the product probably shortening visual freshness and more generally its organoleptic characteristics. Conversely, the RH% trend shown by the PRSTM does not show irregular peaks, but gradual reaching and keeping high humidity values (flat line, Fig. 2), so reducing the impact on the conserved products. The same pattern is observed when the temperature trends of the two systems are compared. A rapid and non-invasive technique able to monitor fish appearance, as image analysis combined with colour metres presented in this work, could be very important for the industrial practices and to meet consumers' preferences. As reported in Table 1, the results obtained with the PLSDA model seem to confirm the feasibility of its application in food bioprocess for the automated identification of freshness status. In fact, it is possible to observe as the mean percentages

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Fig. 2 Trend of percent relative humidity (a) and temperature (b) for the conventional industrial refrigerator (*Ref*) and the innovative PRS™ system (*PRS*), tested during 5 days of conservation. The temperature inside the conventional industrial refrigerator was measured also in contact with ice (*Ref-ice*)



237 of correct classification of the model, sensitivity and sensibility are equal to 100%. This is a promising result leading to
 238 future applications. All fish used to create the model (T_0 and
 239

T_2) were correctly classified as fresh or non-fresh, respectively, while external test individuals (T_1) were all classified as fresh.

Q5t1.1 **Table 1** Results of PLSDA modelling to highlight colorimetric differences between fresh and non-fresh seabream

t1.2	No. of classified elements	60
t1.3	No. of units (y -block)	2
t1.4	No. of LV	4
t1.5	% Cumulated variance x -block	28.29
t1.6	% Cumulated variance y -block	49.43
t1.7	Mean specificity (%)	100
t1.8	Mean sensitivity (%)	100
t1.9	Mean classification error (%)	0
t1.10	Mean RMSEC	0.5028
t1.11	Random probability (%)	50
t1.12	Mean% correlation classification model	100
t1.13	Mean% correlation classification independent test (T_1)	100 as T_0

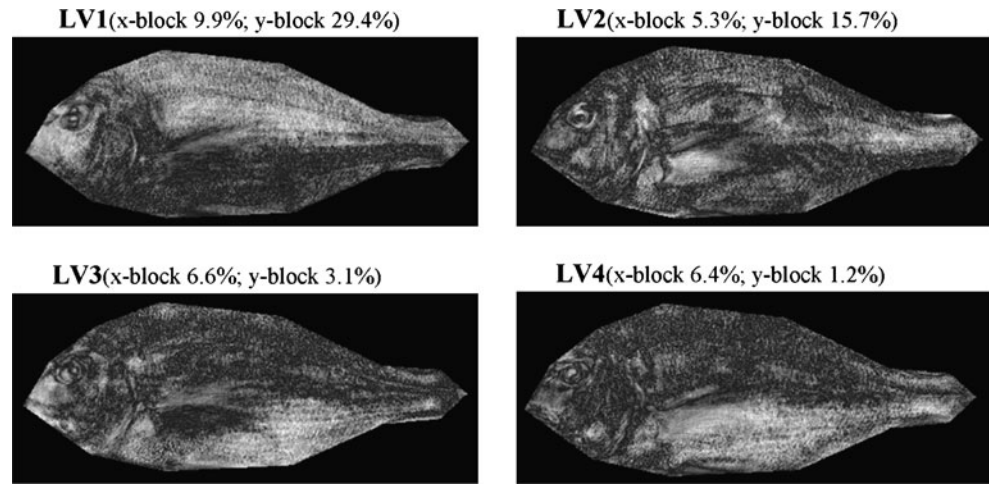
See the text for further explanations

LV latent vector, RMSEC root mean square error of calibration

Figure 3 shows the loading values (contribution) to each LV that each pixel gives to the construction of the model (the white pixels correspond to higher loadings). The first LV, which expresses the main variance on both x - and y -blocks (9.9% and 29.4%, respectively), shows that the most important areas for the freshness discrimination are three: (a) the area below the dorsal fins (Sub-ROI1); (b) the central area below the lateral line (Sub-ROI2); and (c) the anterior region of the cephalic area. The first two areas are easily and quickly identifiable—thanks to the scarcity of organs and to a higher homogeneity of pigmentation pattern—then the third one was excluded from further analysis.

The results of this study shown as MANOVA quantified significant colorimetric differences for the Sub-ROI1 and Sub-ROI2, the two of the most informative areas extracted by the PLSDA model, between fresh (T_0) and non-fresh fish (T_2) and between the four different refrigeration modalities, in all the three RGB components ($p < 0.001$). Consequently,

Fig. 3 PLSDA: scores of the pixels (x-block) for each LV: white intensity is related to the contribution given to the classification



261 those two areas could be used for an instrumental colorimetric evaluation device.
 262

263 The dendrogram in Fig. 4 shows the mean Euclidean distances between the different refrigeration modalities and conservation times. It is possible to observe that the lots closest to the fresh (indicated in the dendrogram as T_0 -ini) are those stored in PRSTM (indicated as T_1 -Prs and T_2 -Prs); then follow the lot T_1 -Out. All the other lots are very distant, including those conserved with the traditional method in a refrigerator with crushed ice (T_1 -Frg and T_2 -Frg). The most distant lots are T_2 -Frz and T_2 -Out. These results show very remarkable colour differences between fresh (T_0) and non-fresh (T_2) fish. It is the first time that the effect of passive refrigeration on fish quality is quantitatively measured, so
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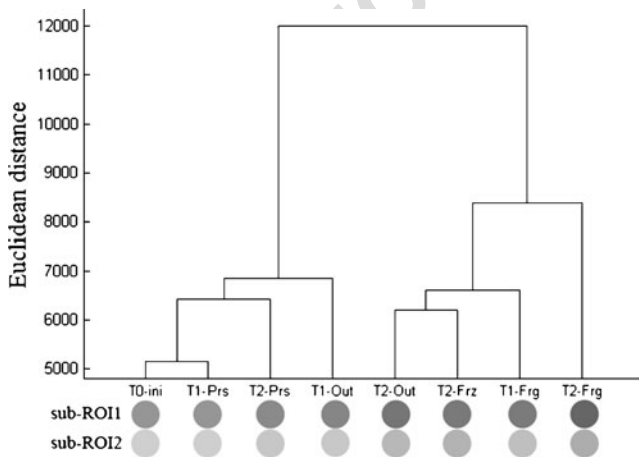


Fig. 4 Dendrogram built from the mean Euclidean distances of the three RGB values decomposed in a single row (129,633 values) between the refrigeration modalities (Frg, Prs, Out and Frz) and conservation times (T_0 , T_1 and T_2). On the bottom side of the dendrogram, the mean colorimetric values obtained on the two Sub-ROIs (Sub-ROI1 and Sub-ROI2; see Fig. 3) are represented. T_0 -ini represents all the samples before conservation

275 showing on a sound and qualitative basis that the fresh
 276 seabream (independently from their experienced life histo-
 277 ry) has a lighter colour with respect to non-fresh. It was also
 278 demonstrated that samples conserved under the PRSTM
 279 show the greatest similarity to the overall coloration pattern
 280 of T_0 fishes, also at the T_2 .

281 The samples weight loss from T_0 to T_2 showed values
 282 significantly lower ($p < 0.005$) for Prs-preserved samples
 283 (0.20%) compared to Frz (0.67%) and Out (0.95%), lower
 284 but not significantly with the Frz (0.42%). Practically, the
 285 use of the PRSTM without ice showed a qualitative fish
 286 aspect better than the one preserved in traditional refrig-
 287 erators, suggesting a higher economic margin.

288 From a technical point of view, the combination of cali-
 289 bration colour (Costa et al. 2009a) with warping (Costa et al.
 290 2009b) (two advanced methods of image analysis) results is
 291 very promising for qualitative analysis of quality aspects,
 292 such as colour and shape. As reported by Olafsdottir et al.
 293 (2004), the European fish industry is still reluctant to imple-
 294 ment methods other than sensory to monitor freshness and
 295 quality of fish products, although general consensus exists
 296 about the importance of various quality attributes and the
 297 need for methods to monitor quality.

Conclusions

298
 299 The proposed imaging method brought out two main
 300 important issues: (1) a sound, qualitative, automated
 301 and non-destructive evaluation of fish freshness based
 302 on visual characteristics by merging different image
 303 analysis techniques, a three-dimensional colorimetric cali-
 304 bration, the morphometric superimposition and PLSDA
 305 modelling and (2) an innovative passive refrigeration
 306 system (PRSTM) is proposed for the best fish freshness
 307 conservation, at least in view of the overall coloration
 308 pattern.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

- Q1. Please check if the author names are presented correctly.
- Q2. Different parts of affiliation have been rearranged. Kindly check if appropriate.
- Q3. The term "GretaMachbeth" was changed to "GretagMacbeth". Please check if appropriate.
- Q4. Please check output of Figure 2 if acceptable, it contains small text below 6 points otherwise, kindly provide figure replacements.
- Q5. Please check if Table 1 is presented correctly.

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