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Original Paper

An electronic tongue for fish freshness analysis using a thick-film array of electrodes

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Abstract. We report on the development of an electronic tongue as potential tool for fish freshness determination. The studies were carried out following the evolution with time on fillet of cultured sea bream (*Sparus Auratus*). The electronic tongue consists of 16 potentiometric electrodes of the type metal, metal oxide and insoluble metal salts. Graphite was also used as electrode. Fish freshness indicators such as texture, pH, colour, microbial analysis, total volatile basic nitrogen (TVB-N) and biogenic amines were also determined versus time. The effectivity of the electronic tongue in the assessment of the evolution with time of fish fillets was evaluated. First the electronic tongue was used to classify samples according to time. Perceptron and adaptive resonance theory were used to discriminate between the data from different days. In a second step, the electronic tongue was used to predict the results obtained from chemical and biochemical analyses by building quantitative partial least square (PLS) models. A remarkable correlation was found between the electronic tongue formed by the 16 simple electrodes and parameters such as total biogenic amines, pH, TVB-N and microbial analysis with correlation coefficients larger than 0.98.

Keywords: Fish freshness; electronic tongue; thick-film technology; potentiometric electrodes

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Traditional food analysis is carried out using a wide range of methodologies based on chemical, biochemical, physico-chemical and microbial principles aiming to determine the concentration or the presence of different compounds that directly participate in the characteristics of food. These traditional analyses offer generally good exactitude and reliability but as a rule are destructive, time consuming and require of laboratories equipped with complex and expensive equipment. Additionally, the analysis should be carried out by specialized personnel and most common classical methodologies are not suitable for *in situ* or *at site* monitoring. Moreover, in many cases, it is more important to have tools for a rapid qualitative analysis of food rather than complex quantitative assays. For instance, in places where foods are prepared, commercialized or stored there is a need of rapid and easy-to-use tools for rapid evaluation of food quality.

In relation to fish, most common methods for fish freshness analysis use diverse physical, chemical and

biochemical methodologies [1]. Also methods for an evaluation of the quality have been developed based on the determination of sensorial attributes such as the so-called Quality Index Method (QIM), in which the aspect of diverse parts of the fish is evaluated and quantified (meat, eyes, gills, skin, etc.), through attributes such as appearance, colour, texture and odour. The QIM method converts these observations in a final numerical output that can be correlated with fish freshness [2]. In order to apply this method of work it is necessary human panellists, well prepared and trained, with all the disadvantages that this circumstance entails (subjectivity, sensorial fatigue, heterogeneity, economic cost, etc.). The introduction of artificial instruments that imitate the human senses is an alternative of interest. One example is the use of the Artificial Quality Index (AQI) that tries to replace the different human senses with corresponding artificial instrument [3]. Thus colorimeters, texture analyzers, electrical impedance and spectroscopic measurements, and image analysis are commonly applied techniques in this protocol.

The electronic tongue concept has its precedent in the electronic nose which consists of an array of gas sensors, showing cross sensibility to several gaseous species [4], coupled with techniques of multivariate analysis [5]. Electronic noses have been applied to the determination of freshness through the AQI coefficient but, as far as we know, electronic tongues have not been applied for this purpose. Among different possibilities for fish freshness monitoring using electronic tongues we focused on the use of potentiometric methods. Potentiometric protocols are based on the measurement of the potential between an active electrode and a reference electrode that are both immersed in the aqueous system of study. This technique is widely used in analytical chemistry through the use of the ion-selective electrodes that respond to the presence of certain analytes. For instance, in case of meat analysis, potentiometric methods have been used for the determination of chloride and nitrate of pork meat products [6, 7]. Although the use of ion-selective electrodes could be an appealing way of monitoring food quality (via sensing of certain species that can vary its concentration with time) we were interested in following a different approach based on the use of rather unspecific electrodes (such as metal electrodes) in an ''electronic tongue'' fashion. The electrodes are expected to respond to a number of species but at the same time could be able to determine, in a qualitative manner, the evolution of complex systems such as meat or fish degradation. In fact, ''electronic tongues'' have been used extensively in the classification of different drinks [8], but to a lesser extent in solid foods [9]. Several types of electrodes have been used in those systems, from PVC-membrane sensors [10] to metallic electrodes [11]. It has been found that certain active components on inks used in thick-film technology respond to the presence of certain chemical species [12] and in fact sets of sensors in thick-film technology have recently been used for the analysis of complex systems such as water samples [13]. Inspired by these studies, it appeared interesting to us to extend these concepts to other matrices and to use thick-film electrodes for the determination and control of food quality. In this context the aim of this paper is to show preliminary studies on the use of an electronic tongue built up with an array of 16 electrodes on thick-film technology for fish freshness analysis.

Materials and methods

Raw material

Fillets of cultured sea bream (*Sparus Auratus*) purchased in a local supermarket were used in the study (one day post-mortem). This specie was chosen because of its high commercial value, availability throughout the year and possibility of controlling the post-mortem time. Additionally, since the used fishes were farmed all of them had very similar characteristics. Additionally, several other works have been reported studying the quality of this kind of fish [14]. Each piece was cleaned, gutted and filleted. Every fillet was analyzed in triplicate for all the analytical determinations except for Microbial analysis (two samples from every fillet) and pH measurements (10 times).

Manufacture of the potentiometric measurement system

The measurement system has been built for this specific application and is formed by several blocks (Fig. 1). The first is an array of

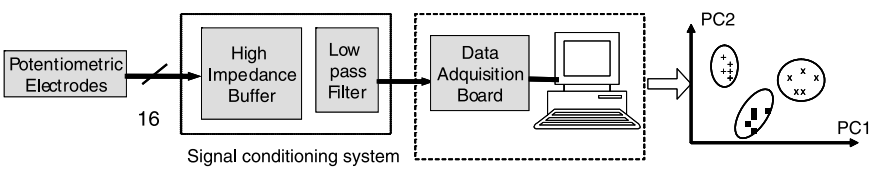

Fig. 1. Schematic diagram of the measurement system

potentiometric electrodes built in thick-film technology, the next block is a signal conditioning system made for two stages, the first one, with a very high input impedance and very low current of polarization is made with electrometric amplifiers LMC6001 (www.national.com) and the next stage is formed by active low pass filter in order to eliminate the noise signals that come from the electrical network. The acquisition and digitalization of the data were made with the PCI-9112 card of Adlink (www.nudaq.com) incorporated in a personal computer and VEE-Pro software (Agilet Technologies, Santa Clara, CA, www.home.agilent.com). In order to make the multivariate analyses the software Matlab 7.0 (Matth Works, Matick, MA, www.mathworks.com) with its annexed programs PLS_Toolbox (Eigenvector Research, Inc. Wenatchee, WA, software.eigenvector.com) and Neural Network toolbox was used.

Thick-film array of electrodes

In order to make the measurements a multi-sensor system formed by 16 electrodes was designed and constructed. The electrodes were prepared using the same procedure than that used in the production of thick-film resistors. The screen-printing process consists of forcing, with a squeegee, inks of different characteristics through a screen onto a surface substrate. In order to manufacture the array, different screens have been designed corresponding to three layers; i.e. Ag–Pd that acts as conductor, the corresponding electrode and an overglace that acts as protector. Heraeus pastes (Heraeus Holding GmbH, Hanau, Germany, www.heraeus.com) have been used for the manufacture. The array has been screened onto an alumina board of dimension of 2×1 inches and thickness 0.3 mm Rubalit 708S, (CeramTec AG Plochingen, Germany, www.ceramtec.com). The electrodes are distributed on the two sides of the board, 8 in each side in array form. The electrodes were prepared from commercially available pastes with different active elements; platinum (Ag/Pt), gold (Au), silver (Ag), graphite (C), silver-palladium (Ag/Pd), Ag/AgCl, copper (Cu) and RuO_2 of resistivity $100\,\Omega\,sq^{-1}$ (RuO_2). The array contains two electrodes of each type. A summary of the pastes used for the array manufacture and the burning temperature in each case is shown in Table 1. We have chosen these materials because they may be sensitive to chemical changes occurring during the spoilage of fish [15]. These electrodes have been used by us in previous studies and we have found that they can respond to certain chemical species. Thus for instance RuO_2 is known to give response to pH [16], noble metals to changes in redox potential [17] and AgCl responds to the presence of chlorides. Additionally, we have found that these systems also act as unspecific sensors in complex media and are suitable materials for the preparation of electronic tongues.

The scheme of the distribution of each electrode in the board is shown in Fig. 2. Each side of the board has nine layouts to place the active pastes. Eight of them are used, and the one in the centre (in black in the figure) is free for the incorporation of a possible reference electrode built in thick-film technology. The conductor tracks transport the electrical signal produced by each electrode towards one end of the board in order to bring the potentiometric signals to the measurement system. Electrodes built with thick-film technology have been recently used to obtain electronic tongues for the discrimination of different types of mineral waters [18] and aqueous solutions [19].

Table 1. Characteristics of thick-film pastes used for electrodes

Active Element	Paste Model	Supplier	Burning (°C)
RuO_2 ($100\,\Omega\,sq^{-1}$)	R8931	HERAEUS	700
Cu	C7257	HERAEUS	850
Ag/Pd	C4248	HERAEUS	850
Ag	C1075S	HERAEUS	850
Ag/Pt	C1076S	HERAEUS	850
C	C2000802D2	GEM	175
Au	G40427D1	GEM	850
AgCl	C61003P7	GEM	80
Protective paste	IP020	HEREAUS	80 (2 h)
Conductive paste	C4081T	HEREAUS	800

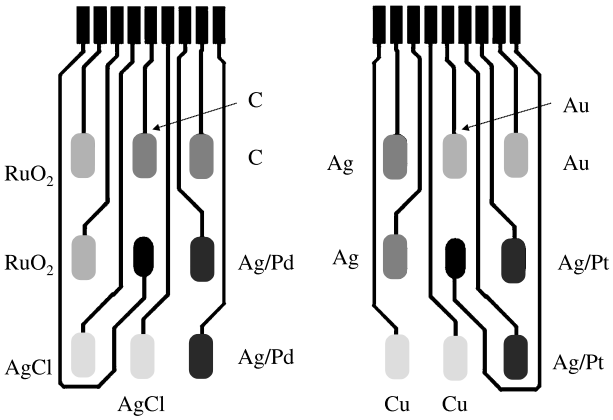

Fig. 2. Electronic tongue design containing and array of 16 electrodes in thick-film technology

The board containing the array of electrodes was placed into a fish fillet and a calomel reference electrode was also placed inside the fish flesh. The set of fish and electrodes was stored at 4 °C during 14 days. Simultaneously, 30 fillets obtained from fishes of the same batch were used for the determination of certain bio-chemical parameters (vide infra).

Analytical determinations

Texture measurement

Mechanical properties were evaluated using a compression test with a TA-XT2 Texture Analyzer (Stable Micro System, Surrey, UK, www.stablemicrosystems.com) controlled by a computer with Texture Expert v 1.22 software®. For this analysis, stored samples obtained from dorsal area were cut in $20 \times 30 \times 10$ mm portions. A shear test was carried out (40 mm penetration at a constant deformation rate of 1.5 mm sec^{-1}) with a Warner Bratzler cell. The fish pieces were placed with the fibers perpendicular to the cutting blade. The maximum force obtained in the analysis (Newtons) was used as an indicator of textural changes throughout the storage of the fillets.

Colour measurement

Each sample was skinned, boned, homogenized and placed in a white plastic cup 1 cm thick to assay colour by surface reflectance. CIE $L^*a^*b^*$ colour coordinates were obtained with a Minolta CM-1000R colorimeter (D65, 10°) (Minolta Co., Tokio, Japan, www.konicaminolta.com).

pH measurement

The pH values were measured by using a digital pH-meter (Crison Instruments, Barcelona, Spain, www.crison.es) by inserting the electrode in ten distinct allocations on every fillet.

TVB-N determination

TVB-N was determined by distillation, with drag steam, of a 2 g sample homogenised in 50 mL distilled water with 0.2 g of magnesium oxide and 1 mL of isopropyl alcohol. The distillation was performed in a Kjeltec rapid distillation unit (Büchi B-316, www.buchi.com) and the distillate was titrated with 0.01 N sulphuric acid. TVB-N: refers to the nitrogen from other sources than proteins.

Biogenic amines determination

HPLC analyses were performed with a Waters Module I plus apparatus equipped with a UV detector (Waters 486, sci-support.com) and a pump (Waters 600). The column, purchased from Waters (Nova pack C18 4 µm (3.9 × 150 mm) was kept at 40 °C. Biogenic amines standards were purchased from Sigma-Aldrich, Poole, Dorset, UK, www.sigmaaldrich.com. The pre-column derivatization reagent was phenylisothiocyanate (PITC) (Fluka Chemie). The mobile phases were: (A) sodium acetate, water and ethanol; (B) acetonitrile, water.

Microbial analyses

Microbial analyses were performed at 0, 1, 2, 3, 4, 5, 6, 7 and 8 days of storage. Ten milliliter of buffer phosphate (BF) (KH_2PO_4 0.25 M solution) adjusted at pH 7.2 ± 0.2 with a NaOH 1 N solution was used for dilutions. After homogenizing in a stomacher for 1 min, a serie of tenfold dilutions was made in BF. Mesophilic counts were performed using Plate Count agar (Merck, Darmstadt, Germany). A volume of 0.1 mL of each dilution was spread thinly on the surface of the agar plates. Counts were done after incubating 2 d at 31 °C [20]. Each sample was analyzed in duplicate, and the microbial analysis of each one was also performed in duplicate, in such a way that for each fillet 4 data were obtained. The results were expressed as log CFU g^{-1}.

Results

Potentiometric measurements

Changes in the redox potential of electrodes such as those used here (i.e metal, metal/metal oxide and metal/metal insoluble salt) is a rather unspecific process and a number of changes in post-mortem fish characteristics can affect to their potential. For instance, small modulations in pH, redox potential or formation of assembled monolayers of certain compounds (e.g. thiol containing molecules in gold and silver electrodes), quimisorption and physisorption processes, etc. can cause significant although unspecific responses. However, and despite this rather unpredictable behaviour we and others have recently reported that arrays of metal-based electrodes could be used as a simple mode to develop electronic tongue like devices. Based on these previous concepts, we believed that the variation of the potential of simple potentiometric electrodes could be a suitable method for the analysis of the evolution of fish stored throughout time.

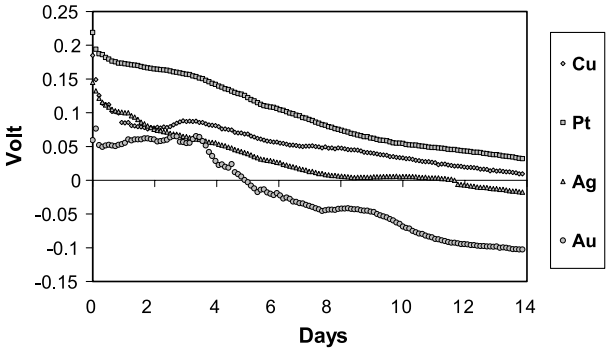

Fig. 3. Potential of selected electrodes from the electronic tongue in contact with fillets of cultured sea bream versus time

In a first step, the evolution versus time of the set of electrodes in contact with cultured sea bream fish filets at 4 °C throughout a total of 14 days was monitored. A selection of the response of certain electrodes versus time in contact with the fish filets is shown in Fig. 3. Clear changes of the potential of the electrodes as a function of the storage time are observed. In most of the cases a permanent decrease in the potential can be seen. The evolution of the measurements bears a resemblance to changes in quality factors reported by other authors and justifies a more insightful study of the relation between potential changes and other fish quality indicators and bio-chemical changes post-mortem.

Multivariate analysis

Spontaneous clustering with PCA

One of the basic characteristics of the electrodes that are part of potentiometric electronic tongues is that their response shows crossed sensitivity [21]. In intricate systems such as food degradation processes a simple and useful technique to obtain conclusions from the large number of experimental measurements is to carry out multivariant analysis. Thus, in order to stress the relationship of the collected signals throughout storage time, PCA studies were carried out. An average value of the signals obtained from the electrodes was calculated every day (from 1 to 14). The values were determined by using 5 representative measurements, obtained every 2 h (a total of 70 measurements were used).

Figure 4 plots PCA for the five different averaged trials taken per day. The data in the PCA plot were mean-centred and the two first principal components

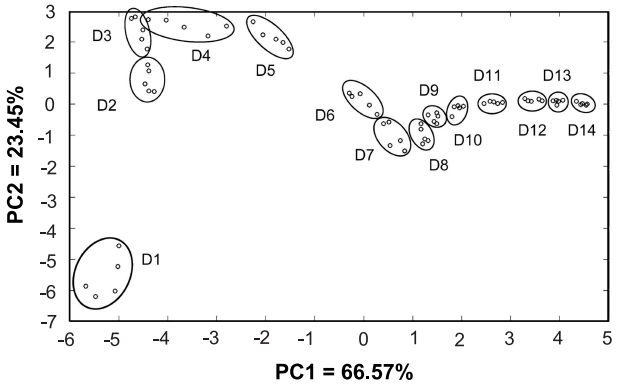

Fig. 4. PCA results for the metallic electrode response. Ellipses cluster together measurements carried out the same day (from *D1* to *D14*)

accounts for 90% of variance. The PC axes are calculated to lie along lines of diminishing levels of variance in the data set. It is observed a clear discrimination with time with a concomitant variation throughout the x-axis; i.e. data from consecutive days are clearly clustered and move from the negative to the positive part of the PC1 axis.

Classification with artificial neural networks

The data can also be analysed using artificial neural networks. These networks need a training phase with a set of measurements and a further phase for validation. Two types of artificial neural networks have been used, so to make a comparison between their results. The analyst is to distribute the measures in three groups, corresponding to the first day (exit A), a set of days 2, 3, 4, 5 and 6 (exit B) and a last third group with the rest of days (exit C). The reason for this classification is a conclusion of the PCA results (Fig. 3) where a distribution in approximate three groups is observed.

The first artificial neural network used was of multilayer perceptron type (MLP) feed-forward formed by an output layer with 3 neurons, one for each group A, B y C, 16 inputs for each electrodes and a hidden layer formed by 7 neurons. Each neuron has a transfer function of *logsig* type. The neural network used has an output layer with neurons with a *logsig* transfer function, that generates an analogical result between 0 and 1, but nonlinear. The interpretation of the results is made so that output values over 0.5 assign an output of 1, and 0 in the other way around. The transfer function of the neurons of the layer hidden is also of the type *logsig*. The inputs are combinations of the potential from different electrodes, whereas the ideal exit is a codification ''one-to-three'', in which the correct classification is denoted with a 1 and the two inactive classes by a 0. The implementation of the neural networks was made by means of Matlab software and its annexed program Neuronal Network toolbox.

For the training and validation of the neuronal network the method ''leave-one-out'' was applied using an array formed by the 70 measures (5 measures in 14 days) from 16 electrodes. This method allows the same measurements to serve for both the training and for the evaluation, and it consists of taking all the measures available except a vector (16 electrodes) to make the training and the remaining vector for the evaluation. This task is repeated changing the vector of measures that was left for the evaluation. Following this protocol many evaluations are made and it is verified if each evaluation agrees with the theoretical exit. With all these calculations a rate of success is obtained according to the coincidence of the real exit with the theory. The total rate of success is: 63/70 (90%).

In order to try to improve the results of the classification of the electronic tongue, another type neuronal network denominated fuzzy ARTMAP was also used. These networks are formed by adaptive resonance theory (ART) networks to achieve supervised learning [22]. Fuzzy ARTMAP is a self-organizing supervised classifier that in general is better at training than other protocols. In fact from a practical point of view fuzzy ARTMAP works very well when there are few samples to classify [23]. The network has been implemented in-house using function macros (m) from basic functions of Matlab. As in the previous network, this network has 16 inputs corresponding to the 16 electrodes. With this method a remarkable rate of success of 100% is obtained.

Chemical and biochemical measurements

pH and TVB-N evolution

Changes in pH and TVB-N in the fish samples throughout the storage time can be seen in Fig. 5. The initial pH was close to 5.8, which is a typical value in very fresh products, and is achieved after a decrease in the pH of the muscle as a consequence of the enzymatic activity occurred just at the very beginning of the post-mortem stage. The pH values were within the range observed in other studies. The pattern

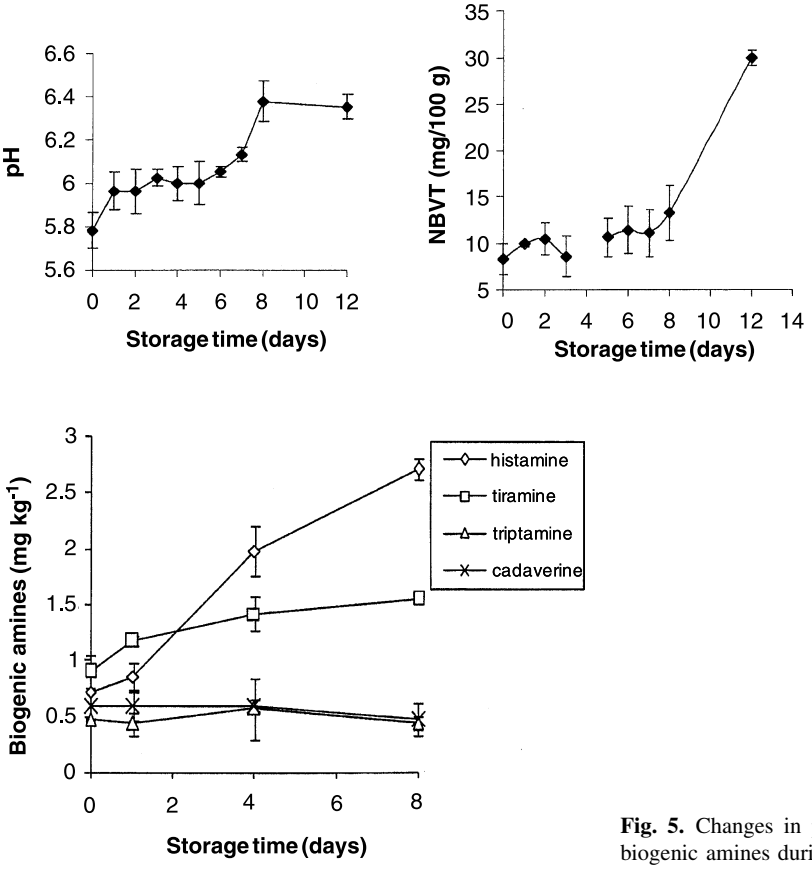

Fig. 5. Changes in pH, Total Volatile Basic Nitrogen (*TVB-N*) and biogenic amines during storage time

in the pH changes was the same than that observed for TVB-N (Fig. 5) which is also similar to that observed by Lougovois et al. [24]. The changes found in the mentioned parameters points to three stages, the first corresponding to days from 0 to 2 (highly fresh product), the second from day 3 to 6, and at last a third period from 7th day, corresponding to a degraded product.

Changes in biogenic amines and microbial load

Changes in biogenic amines can also be seen in Fig. 5. An increase with the storage time was observed for histamine and tiramine, while triptamine and cadaverine remained nearly constant in the studied period. It is well known that the biogenic amines are originated through the decarboxylation of amino acids as a consequence of the activity of certain microorganisms [25]. In fact a similar pattern was observed in the changes of total viable counts throughout the storage time. The average of total viable counts (TVC) during the first 5 days was around 10^4 CFU g^{-1}. The spoilage levels of 10^7 colony-forming-units (CFU) g^{-1} were exceeded after 7 days, reaching 10^8–10^9 CFU g^{-1} at the end of the trial [26].

Texture and colour changes

Texture and colour of fish are two physical properties that have been related with the evolution of fish freshness [27]. As regards the maximum force obtained in the shear test, a clear plateau was observed for the first 6 days, with an average value of 19 N, while a clear decrease in the maximum force was observed at 8th day of storage (11.8 N). When the storage time increased the brightness (L*), a* and b* values increased. The mucus appearing on the surface contributes to the increase in lightness while the chemical and enzymatic browning of the fish flesh contributed to the decrease in the red (a*) and green values (b*).

Potentiometric results and chemical and biochemical analysis

After the potentiometric measurements using a set of electrodes on filets of cultured sea bream (*Sparus*

Auratus) and the physical, chemical and biochemical analysis carried out, in this section we attempted to establish a relationship between the analytical and the electronic tongue measurements by using partial least squares (PLS) procedures. PLS is a multivariate projection method for modelling a relationship between dependent variables (Y) and independent variables (X). The principle of PLS is to find the components in the input matrix (X) that describe as much as possible the relevant variations in the input variables and at the same time have maximal correlation with the target value Y, giving less weight to the variations that are irrelevant or noisy [28].

A fold training/validation approach was used for each parameter. The leave-one-out technique was applied [29]. The data input to the models were the mean-centred responses of the 16-element sensor array. By using those data, a predictive model was built, training the model with the rest of the data. Initially, each model was fully cross-validated to determine the number of factors (latent variables) to be used. The PLS method was employed to correlate potentiometric measurements using the set of potentiomentric 16 electrodes and the parameters texture, colour, pH, TVB-N, microbial analysis and biogenic amines. Figure 6 shows the predicted versus the actual values for texture, total amines, TVB-N and microbial analysis. Ideal prediction would place the points along the diagonal line when both predicted and actual data are the same. The results of this PLS analysis are also summarized in Table 2, where accuracy was estimated using three parameters obtained from the fitted equation: the correlation coefficient between predicted and experimental values, the slope and intercept. The closer to 1 are the slope and correlation values and the closer to 0 are the intercept, the better (more accurate) is the calibration model. As can be seen in the table good correlations were found in most cases especially for that between potentiometric data and total amount of biogenic amines. In contrast a worse correlation with the potentiometric data was found for parameters such as colour and texture with correlation coefficients lower than 0.9. For certain parameter such as colour the experimental data show important discontinuities versus time and for this reason the correlation with the potentiometric signals is low.

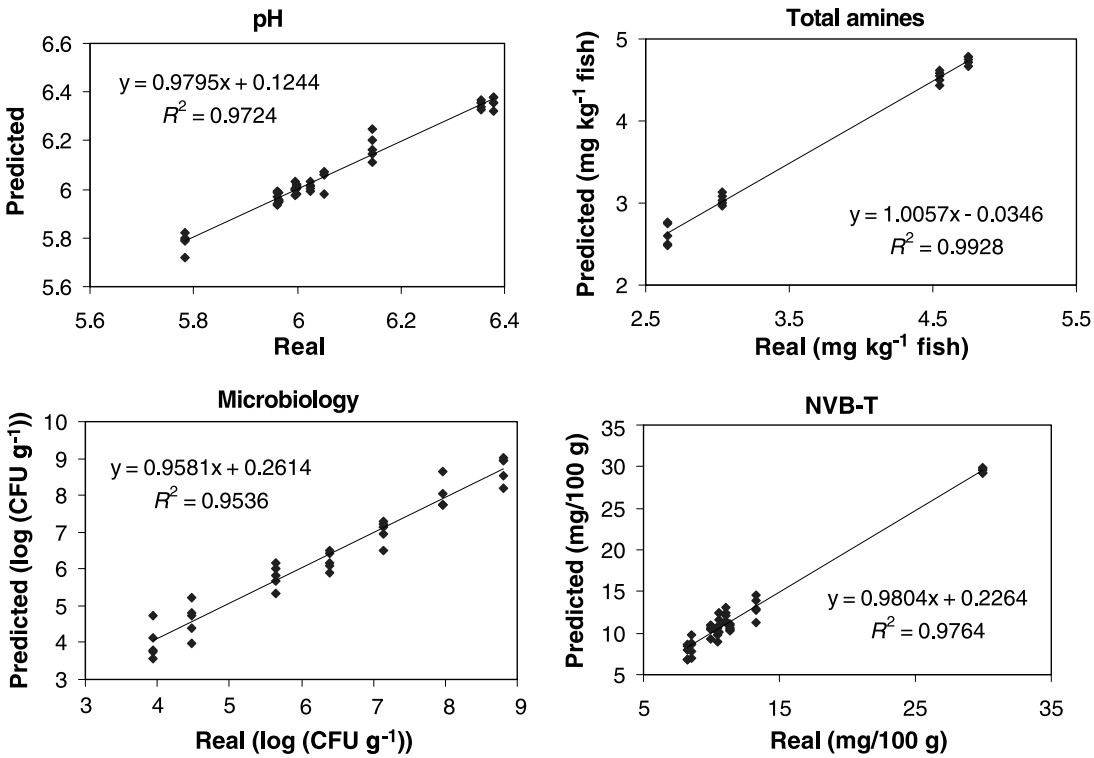

Fig. 6. Predicted versus actual values of certain parameters related with fish freshness given by PLS models

Table 2. Prediction results on some quality parameters

Quality indicator	No. Latent Var.	Correlation Coef.	Slope	Intercept
Texture	10	0.900	0.96	0.59
Colour				
Brighty	9	0.815	0.81	7.65
a	14	0.884	0.85	0.19
b	14	0.898	0.89	0.19
Cab	12	0.785	0.83	0.52
Hab	8	0.897	0.86	20.07
pH	8	0.972	0.98	0.12
TVB-N	10	0.976	0.98	0.23
Microbial analysis	16	0.954	0.96	0.26
Amines				
Histamine	4	0.991	1.00	0.00
Tyramine	7	0.990	1.00	0.02
Total amines	5	0.993	1.00	0.03

Conclusion

The correlation between certain degradation parameter and changes in the potential of a set of electrodes has been used as a simple yet accurate method for the evaluation of fish freshness. This electronic tongue that uses the potentiometric signal of 16 rather unselective electrodes have proven to be dependent on the storage time of whole sea bream. The potentiometric method we report herein is fast, non-destructive and low-cost and we suggest it might be applied for *in situ* and *at site* fish degradation monitoring in a wide range of situations. The effectiveness of the electronic tongue in the assessment of the evolution with time of fillets of cultured sea bream (*Sparus Auratus*) was evaluated by two different methods. First we observed that the electronic tongue could be used to classify the evolution of fillets of cultured sea bream with time. The system input the data from the potentiometric measurements into a fuzzy ARTMAP classifier. The system could discriminate between the data from different days. In a second step the electronic tongue was used to predict the results obtained from chemical and biochemical analysis by building quantitative PLS models. A good remarkable correlation was found between the electronic tongue formed by 16 simple electrodes and parameters such as total biogenic amines, pH, microbial analysis and TVB-N with correlation coefficients larger than 0.95. We believe that this procedure can be a complement to classical sensory analysis for freshness evaluation and an alternative to other high-cost and time-consuming methodologies.

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