Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 681–688
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical Methods

Freshness monitoring of sea bream (*Sparus aurata*) with a potentiometric sensor

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Received 4 May 2007; received in revised form 13 September 2007; accepted 11 October 2007

Abstract

Freshness in one of the main quality attributes for fish commercialization and consumption. The traditional method for fish freshness evaluation is sensory analysis. However, instrumental methods such as electrical, texture and colour measurements, image analysis, VIS spectroscopy and electronic noses have been widely studied as objective alternatives. Each of these methods has advantages and disadvantages, but none of them can be universally proposed for defining and measuring fish freshness.

This work evaluated the correlation of potentiometric measurements, obtained with gold and silver electrodes, with physicochemical, microbiological and biochemical analyses of sea bream stored under refrigeration. Results showed a strong correlation of the potentiometric measurements with the determined changes in fish, and an important correlation with the K_1 index, dependent on the nucleoside degradation, which is used as a good indicator of *post-mortem* time and freshness.

Keywords: Fish; Freshness; Potentiometric method; Fish quality

1. Introduction

Fish quality is a very complex concept, which includes nutritional, microbiological, biochemical and physicochemical attributes related to this term.

Freshness is considered as one of the most important parameters of fish quality in most markets (Ólafsdóttir et al., 1997). The state of freshness can be described by various properties of the fish which can be assessed by various indicators (Bremner & Sakaguchi, 2000) that are dependent on different biological and processing factors (Botta, 1995).

Once fish is dead, a number of degradation reactions begin, contributing to diminish its freshness. Some of those reactions are chemical, mainly the oxidation of organic compounds; others are biochemical (proteolysis and lipolysis) (Surette, Gill, & Leblanc, 1988) and result from microbial metabolic activity (Sikorski, 1990). In addition, microorganism growth implies an overall decrease in quality and safety (Liston, 1980).

The degrees of fish freshness can influence, not only quality attributes for fresh consumption, but also fish behaviour during further processing (Barat et al., 2006). There are some measurements directly related to fish freshness; viable bacterial counts (Kaneki et al., 2004), concentration of ATP and its breakdown compounds (Karube, Matsuoka, Suzuki, Watanabe, & Toyama, 1984; Surette et al., 1988) and volatile amines originating from the decarboxylation of amino acids. All the analytical techniques currently used to study fish freshness have proven validity.

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doi:10.1016/j.foodchem.2007.10.034

Nevertheless, their widespread application in the industry is currently limited because they are relatively expensive, time-consuming, require highly skilled operators and are not suitable for on-line monitoring. One important consequence is that current methods cannot simultaneously evaluate correct fish freshness when fish is sold and therefore they are designed for in-laboratory use only. For this reason, there is a pressing need to develop new, rapid, non-destructive and relatively low-cost analytical techniques, which can be applied in the factory or in the market as on-line sensors for monitoring fish freshness.

Some of the instrumental methods which can be used for measuring fish freshness are the following:

- Electrical measurements (e.g. conductance and capacitance) (Oehlenschläger, 2003) to detect changes after fish death causing disruption of the cell membranes by autolytic spoilage.
- In some cases colour change has been correlated with changes in fish freshness (Schubring, 2003). There is a tendency to change from reddish to brownish colour, and the changes in fish flesh appearance go from an initial bluish, translucent, smooth and shining appearance to an opaque aspect.
- Image analysis to calculate the colour and turbidity of the mucus on the skin and the coarseness of muscle fibres on the surfaces of fillets (Kroeger, 2003).
- VIS spectroscopy to measure spectra changes, which seem to be related to the degree of spoilage during chilled or frozen storage (Heia, Esaiassen, & Nilsen, 2003).
- Texture has been found to change with fish freshness evolution. Careche et al. (2003) observed that measurements of firmness and parameters extracted from the stress relaxation curves correlated well with sensory textural attributes, such as firmness, dehydration or water loss.
- Electronic noses, which can monitor the onset of spoilage of fish by detecting volatile degradation compounds (Amari et al., 2006; Ólafsdóttir, Di Natale, & Macagnano, 2003).

Nevertheless, it has been observed that none of the above mentioned techniques are useful on all occasions (Ólafsdóttir et al., 2003).

Potentiometry is a simple method that is based on the measurement of the electric potential of electrodes vs a reference electrode.

Potentiometric techniques have been used in analysis and discrimination of beverages (Ciosek, Brzózka, & Wróblewski, 2004), but to a lesser extent for solid foods with high moisture, e.g. meat and fish (Legin, Rudnitskaya, Selenev, & Vlasov, 2002). Recently, potentiometric techniques using metallic electrodes or metal oxides have been used for the qualitative analysis of natural waters (Martínez-Máñez, Soto, García-Breijo, Gil, Ibáñez, & Gadea, 2005; Martínez-Máñez, Soto, García-Breijo, Gil, Ibáñez, & Llobet, 2005) and potentiometric methods have been used to determine chloride and nitrate in pork meat products (Pérez-Olmos, Herrero, Lima, & Montenegro, 1997) or for the evaluation of freshness, where the signals collected with Pt, CuS and Ag_2S electrodes were related, by means of multivariate analysis techniques, to viable bacterial counts (Kaneki et al., 2004).

Inspired by these recent papers reporting the use of potentiometric measurements for foodstuff analysis and discrimination, we report herein, the results obtained from the use of Au and Ag wires for the analysis of the changes of sea bream stored under refrigeration. Discussion of the suitability of the method for fish freshness determination and the correlation of the potentiometric measurements obtained with the gold and silver electrodes with the evolution of certain physicochemical, microbiological and biochemical parameters, complete the issues of the paper.

2. Materials and methods

2.1. Raw material

Cultured gilthead sea bream (*Sparus aurata*) were used in our work. The study began after 12 h of *post-mortem* ice storage. The whole fishes were headed, eviscerated, washed thoroughly of blood, and filleted prior to the determinations.

In the preliminary study, three whole fillets were used. The fillets were stored inside a plastic container at 4 °C for a total of 15 days. Potentiometric measurements were obtained throughout the storage time in order to explore the feasibility of the use of silver and gold electrodes to determine the evolution of fish freshness.

In the following study, working with the minced samples, 15 fishes (30 fillets) were used. All the obtained fillets were minced for 1 min by means of a Moulinex® DJE215 grinder and the homogenized mixture was distributed within three containers. All of the fish samples were stored in a cold chamber at 4 °C for a total of 7 days. Every day, the total viable counts, IMP, inosine, hypoxanthine, pH and potentiometric values (silver and gold electrodes) were determined in each sample. All the measurements were done in triplicate on every sample.

2.2. Analytical determinations

pH was determined in samples throughout the storage time by using a puncture pHmeter (micro pH 2000, Crison®).

2.3. Microbiological analysis

Tenfold dilutions in 0.1% peptone water were prepared from each sample obtained from every container at every measurement day ($n = 3$) and 1 ml aliquots were plated in duplicate. Aerobic counts were determined by using Plate

Count Agar (Merck). Duplicate pour plates were prepared per dilution and incubated at 28 °C for 48 h.

2.4. ATP breakdown compounds

The ATP-related compounds, consisting of inosine 5′-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx), were determined by HPLC. The extraction procedure was similar to that described by Burns and Kee (1985). Five grams of each sample were homogenized with 50 ml of 0.6 M of cold perchloric acid for 4 min at 4 °C by using a masticator (IUL Masticator, Barcelona, Spain). The obtained extract was centrifuged at 10,000*g* under cold conditions (4 °C) for 20 min. The supernatant was filtered through glass wool and neutralised by adding solid potassium carbonate. The neutralised extract was kept in ice for 5 min and then centrifuged at 12,000*g* in a refrigerated microcentrifuge for 10 min. The supernatant was stored at −28 °C prior to analysis.

An HPLC, model 1050, equipped with a diode array detector was used (Agilent Technologies, Barcelona, Spain). Nucleotides were separated in a LiCrospher 100 RP-18 column (250 × 4 mm) (Agilent Technologies) by using a gradient between two solvents which were different for the Hx and for both Ino and IMP analysis. Thus, 0.1% of trifluoroacetic acid (TFA) in water (A) and 0.085% of TFA in 60% acetonitrile in water (B) were used for the Hx analysis and 0.01 M dipotassium hydrogen phosphate buffer, pH 4.5 (A) and 60% acetonitrile in water (B) were used for the Ino and IMP analysis. In both cases, the gradient was from 2% to 7% solvent B in 15 min, followed by a 10 min washing step with 100% solvent B. Before a new analysis, initial conditions (2% B) were maintained for 15 min. Flow rate was 0.9 ml/min and separation was achieved at ambient temperature. Injection volume was 20 μl. ATP-related compounds were monitored at 254 nm and their spectral signal in the range 190–350 nm were obtained to assure peak identification. Quantification was performed by an external standard method.

The K_1 was calculated according to Karube et al. (1984) as follows:

$$K_1(\%) = \frac{\text{Ino} + \text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}} \times 100 \quad (1)$$

where IMP is inosine 5′-monophosphate; Ino, inosine; Hx, hypoxanthine.

2.5. Potentiometric measurements

2.5.1. Aqueous solutions

The responses of gold and silver electrodes to changes in pH were studied using buffered solutions at pH 4, 7 and 9. The responses of the gold and silver electrodes to changes in the redox potential were studied using as standard solution of the quinine/hydroquinone couple in water. As the redox potential of this quinine/hydroquinone couple is also pH-dependent, the response of the electrodes was measured at pH 4, 7 and 9. The responses of the gold and silver electrodes to the thiol-containing amino acid cysteine were determined by titration procedures of buffered aqueous solutions (pH 7.5, 0.01 mol dm^{-3} HEPES) containing the corresponding electrode with a 0.01 mol dm^{-3} solution of cysteine in water.

2.5.2. Fish samples

Potentiometric measurements were accomplished by means of three gold and silver electrodes (3 cm long and 0.8 mm of diameter) and a calomel reference electrode. The electrodes were previously sterilized and inserted at every sampling day in the whole or minced fish, and the potential values were obtained when the signals were stabilized.

The signals were acquired and processed by means of a PCI-9112 *Adlink* acquisition board, connected to a PC. To achieve a suitable signal, the measurements were carried out using a high impedance input buffer with LMC6001 electrometric amplification circuits, input impedance larger than 1 TΩ and a very low bias current (25 fA). Although the output impedance of metallic electrodes is quite small, it is necessary to make the measurements with a very high input impedance, and thus to observe the conditions of potentiometric measures that it forces to measure with current zero. In a second step, a Butterworth second order filter with a cut-off frequency of 1.2 Hz was used in order to remove the electric noise signals coming essentially from the electrical network (50 Hz). The software for visualization and data storage was HP-VEE Pro®.

2.6. Statistical analysis

The differences between the assayed samples were analyzed by using ANOVA at a significance level of 95%. Correlation equations of the potentiometric measurements and the analytical determinations were obtained by using the least-squares method. The statistical analysis were done by means of the software Statgraphics, version 5.0; Manugistics Inc., Rockville MD, USA.

3. Results and discussion

3.1. Preliminary results with whole fish samples

The evolution of the average value of the potential of the gold and silver electrodes in contact with whole fish, throughout a total of 15 days at 4 °C, can be seen in Fig. 1 (with standard deviation bars). A similar pattern was observed for both electrodes; a stable period at the beginning, followed by a fast decrease in the values, and finally a tendency to stabilize the signal after a long storage time. The evolution of the measurements resembles the changes in the quality factors described by other authors (Di Natale et al., 2001), and justifies a more profound study of its relationship to other fish quality indicators.

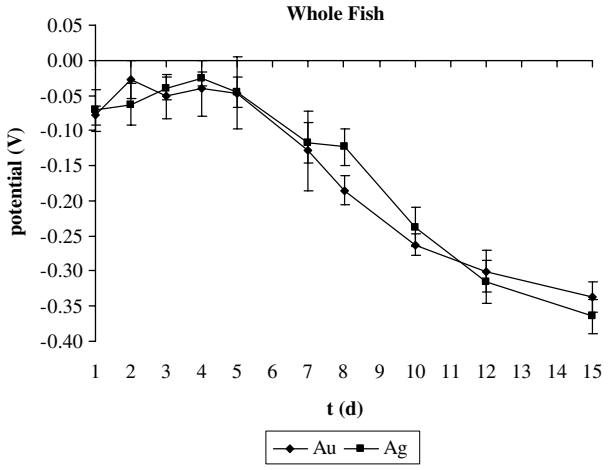

Fig. 1. Measures of the gold and silver electrodes obtained with whole fish throughout the storage time at 4 °C.

Change in the potentiometric signal of gold and silver electrodes is a rather unspecific process and, in fact, a number of modifications in *post-mortem* fish characteristics can affect the final observed potential of the electrodes. Thus, it is well known that certain metallic electrodes show responses to relatively small changes in the pH and/or redox potential in aqueous solutions (Hsueh, Liu, Henry, & Freund, 1999). For instance, it has been reported that the redox potential of fish changes throughout storage time (Huss & Larsen, 1979); i.e. the redox potential is positive and constant while the fish is acceptable for human consumption, and decreases rapidly when fish spoils. Another chemical process that can be observed in gold and silver electrodes is the reaction with thiol-containing derivatives that tend to form self-assembled monolayers on their surface (Flink, van Veggel, & Reinhoudt, 2002). Thus, it is known that thiol-containing amino acids can be anchored to the surface of gold by means of covalent bonds (Gooding, Mearns, Yang, & Liu, 2003) and some studies have been published indicating the detection of the thiol-containing cysteine by using gold electrodes (Possari, Carvalhal, Mendes, & Kubota, 2006; Wang & Du, 2002).

These potentiometric responses can be observed in Fig. 2, that plots the variation of the potential of silver and gold electrodes caused by changes in aqueous solutions of the pH, the redox potential and the concentration of cysteine.

Clear relationships can be observed. In the case of aqueous solutions containing cysteine, the response is very clear (Fig. 2) and shows a near Nernstian behaviour, with a slope of 61 mV/decade.

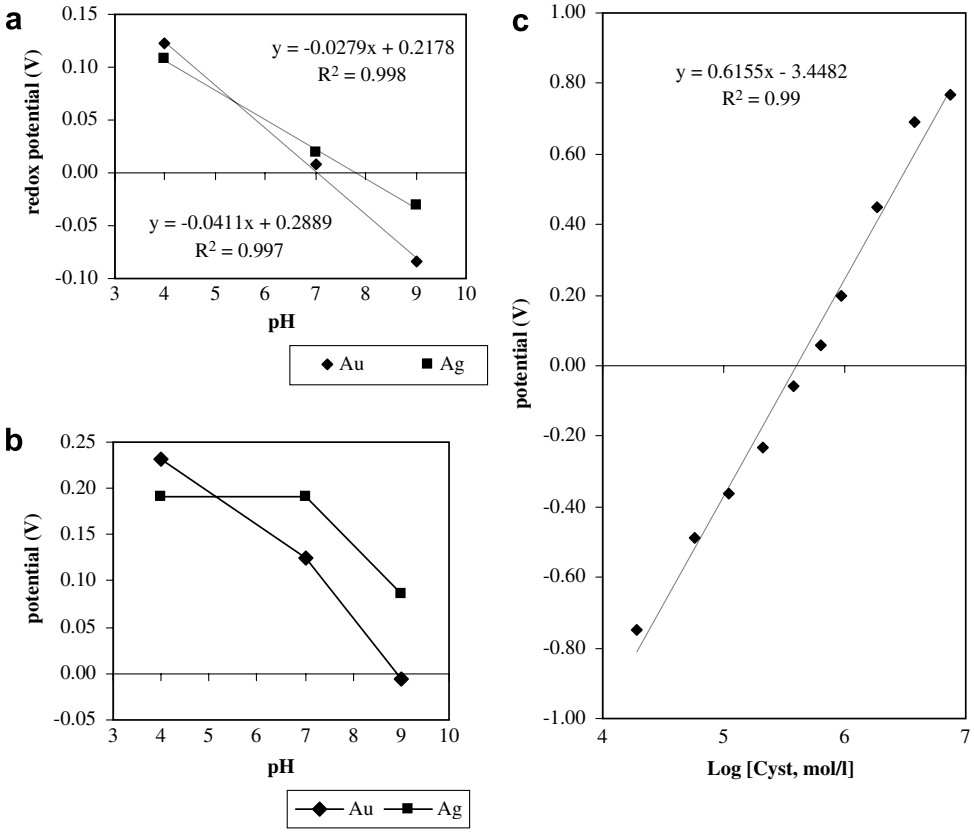

Fig. 2. Variation of the potential of gold and silver electrodes as a function of (a) changes in the pH, (b) changes in the redox potential (via changing the pH of a quinine/hydroquinone solution) and (c) changes in cysteine concentration in water.

3.2. Results on minced fish

3.2.1. Potentiometric measurements

Punctual measurements obtained every day with gold (Au) and silver (Ag) electrodes are shown in Fig. 3. Average values of 9 measurements (3 electrodes applied to each of the 3 batches employed in the study) are given (with standard deviation bars). A continuous fall in the potential throughout storage time can be observed for both electrodes. A possible explanation for the observed behaviour would be the interaction of the electrodes with the compounds appearing during the fish flesh degradation as a consequence of autolytical, chemical and microbiological activity. Although the gold and silver electrodes exhibited the same pattern, the initial values were different, the signals converging as the storage time advanced. Significant differences in the potential values of both electrodes were observed until the 7th day of storage. The change in the potential of the gold and silver electrodes throughout the storage time would be a consequence of the combined changes in the fish characteristics, such as pH, redox potential and protein degradation.

It can be observed that the minced fish exhibits a faster degradation pattern, when comparing the potential values obtained with the whole fish, probably as a consequence of the initial degradation due to the mincing process. The different values in the initial potential registered by the Ag and Au electrodes should be studied in further works.

3.2.2. Microbiological analyses

The averages of total viable counts (TVC) at the beginning were very high (close to 10^5 CFU g^{-1}), probably due to the initial high counts as a consequence of the mincing operation. The spoilage level of 10^7 colony-forming-units (CFU) g^{-1} (Capell, Vaz-Pires, & Kirby, 1997) was exceeded after 4 days, reaching 10^8–10^9 CFU g^{-1} at the end of the trial (Lougovois, Kyranas, & Kyrana, 2003).

A high degree of correlation was observed between TVC and the potentiometric measurements obtained with the gold electrode

$$\text{TVC (CFU g}^{-1}) = 1.23 \times 10^8 - 5.3 \times 10^9 \cdot \text{Au (V)} \quad (R^2 = 0.885) \qquad (2)$$

3.2.3. Changes in pH

Mean pH measurements over the period of storage are shown in Fig. 4. The increase in pH can be associated with the state of rapid spoilage of the fish. The pH values were within the range observed in other studies (Parisi, Franci, & Poli, 2002). The pattern in the pH changes was the same as that observed for the total viable counts and in the same way as observed by Kyrana, Lougovois, and Valsamis (1997); a significant increase was observed until day 4.

A high degree of association was observed between pH of the flesh and the potentiometric measurements obtained with the silver electrode

$$\text{pH} = 5.93 - 6.52 \cdot \text{Ag (V)} \quad (R^2 = 0.82) \qquad (3)$$

3.2.4. ATP-related compounds

The evolution of IMP, Ino and Hx concentrations during the storage of raw cultured sea bream in ice is shown in Fig. 5. ATP, ADP and AMP, which are expected to drop rapidly in the first hours *post-mortem*, were not analyzed. Indeed, the conversion of ATP to IMP is usually achieved within 1 day *post-mortem* and is presumed to be totally autolytic (Hiltz, Dyer, Nowlan, & Dingle, 1972).

In this study, the level of IMP was over 3.3 μmol/g on day 1 and then decreased to negligible values on day 7. Ino increased slightly from 1.6 to 2.0 μmol/g from the first to the fourth day and then decreased rapidly. Hx, which has been recognised as an accurate indicator of freshness in many fish species (Burns & Kee, 1985), increased from the first to the fifth storage day, reaching values of 1.5 μmol/g. The Hx increase is due to both autolytic and microbial enzymes (Surette et al., 1988). The use of Hx content, without any other index of freshness quality, may be misleading whenever a fish has been processed during the latter stages of its storage life (Botta, 1995). As Hx can be oxidized to xanthine, which in turn can be oxidized to

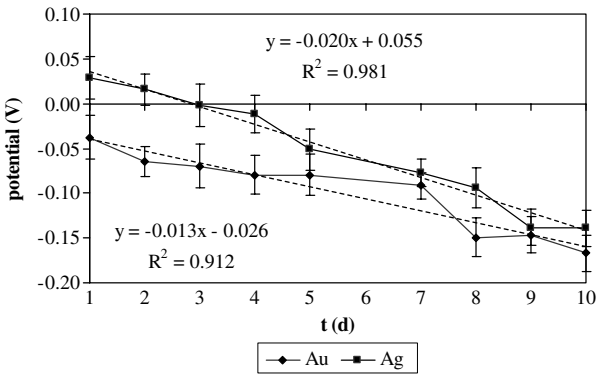

Fig. 3. Variation of the potential (with standard deviation bars) of Au and Ag electrodes throughout the storage time of the minced sea bream.

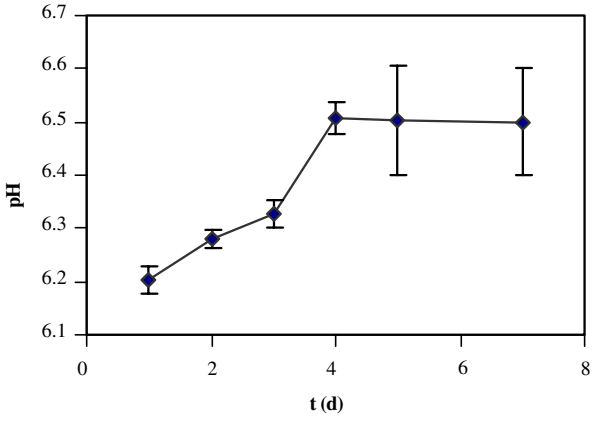

Fig. 4. Changes in pH of minced cultured sea bream throughout the storage time at 4 °C.

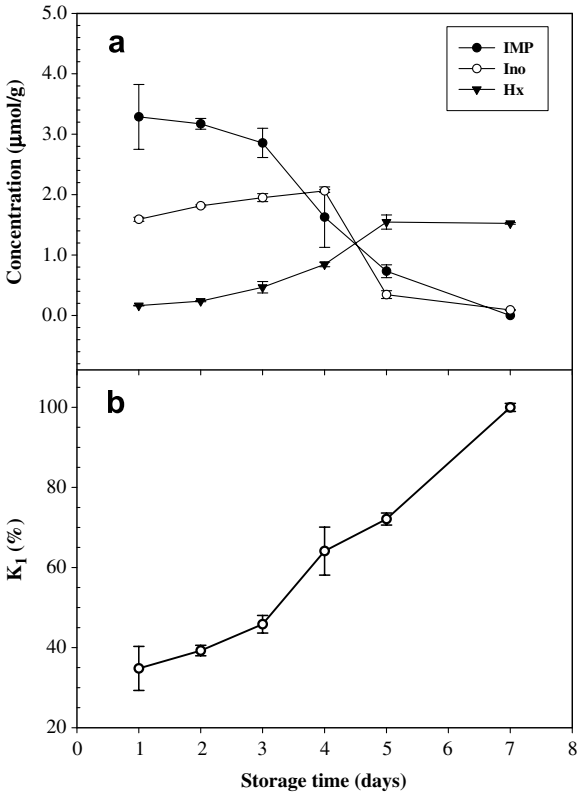

Fig. 5. Changes in concentration of ATP breakdown compounds (a) and K_1 values (b) in minced cultured sea bream stored at 4 °C (with standard deviation bars). Values shown are the means of triplicate measurements.

uric acid, its content can reach a maximum during chilled storage and then start to decline. In this study, the increase of Hx stopped at day 5.

The speed of these degradation and formation reactions depends on several factors, including the storage conditions. So, a more rapid Hx increase was observed in herring held in ice than in herring held under carbon dioxide (Özogul, Taylor, Quantick, & Özogul, 2000). The formation of Hx has been reported to vary considerably within a given species but also within an individual fish, as its formation may be greater in red muscle than in white muscle (Murata & Sakaguchi, 1986). The use of a single ATP metabolite as a freshness indicator across species has not been successful because of the wide diversity in the patterns of nucleotide metabolism from one species to another (Dingle & Hines, 1971). A recent review on the kinetics of degradation of IMP, including the initial concentration of IMP, Ino and Hx in many fish species before chilled storage, has been published by Howgate (2006).

The concept of the *K*-value as a freshness indicator was introduced by Saito, Arai, and Matsuyoshi (1959) and the modified K_1 value proposed later by Karube et al. (1984) have provided a better indication of the loss of freshness in ice-stored fish. As mentioned above, ATP, ADP and AMP are almost completely converted to IMP within 24 h *post-mortem* (Jones, 1965) and the two freshness parameters are comparable after this time. Fig. 5b shows an almost linear increase in the K_1 value of sea bass during storage in ice. This increase is significant from the third day of storage.

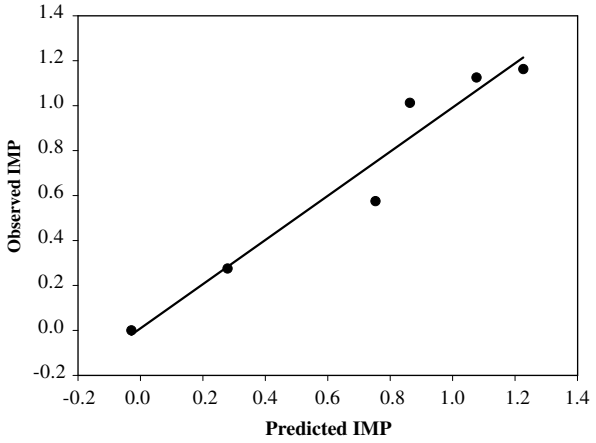

Fig. 6. Values of chromatographically analyzed IMP concentrations represented vs the predicted values obtained with Eq. (5).

3.2.5. Correlations with metal sensors

IMP, Ino and Hx were correlated with the signal emissions by both materials, gold (Au) and silver (Ag). The following correlations were obtained:

$$\text{Hx (mg/g)} = 0.15 - 3.94 \cdot \text{Au (V)} \quad (R^2 = 0.94) \tag{4}$$

$$\text{IMP (mg/g)} = 0.86 + 11.51 \cdot \text{Au (V)} \quad (R^2 = 0.95) \tag{5}$$

$$\text{Ino (mg/g)} = -0.15 - 5.74 \cdot \text{Ag} + 4.73 \cdot \text{Au (V)} \quad (R^2 = 0.94) \tag{6}$$

As an example, the values of chromatographically analyzed IMP concentrations are represented vs the predicted values obtained with Eq. (5) on sensors signals (see Fig. 6). As can be observed, both groups of results showed a very good correlation. This implies that both sensors are measuring very acceptable values, close to those obtained through conventional chromatographic analysis. The advantages are simplicity and the save of cost and time needed to carry out the analysis.

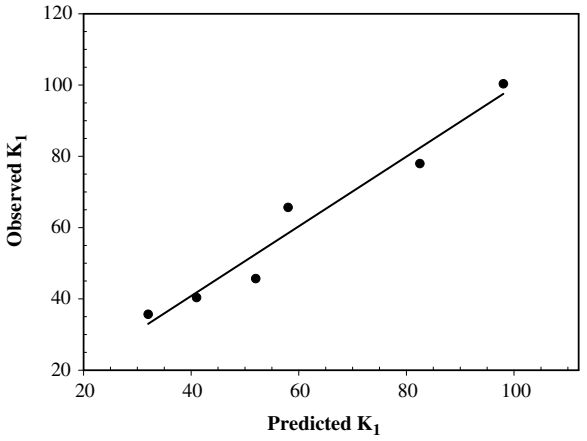

Fig. 7. Values of K_1 index obtained with the chromatographically analyzed IMP, Hx and Ino concentrations represented vs the predicted values obtained with Eq. (7).

Similar results were obtained for the K_1 index, as shown in Fig. 7. The correlation of K_1 index with both materials is as follows:

$$K_1 = 47.58 - 45.64 \cdot \mathrm{Ag} - 597.96 \cdot \mathrm{Au} \quad (R^2 = 0.96) \qquad (7)$$

The correlation in this case was also very good. The predicted K_1 values obtained with Eq. (7), based on sensors signals, agreed very well with those obtained by chromatographic analysis, once again confirming the feasibility of this sensor for a real, easy, rapid and effective detection of fish freshness.

4. Conclusions

The correlation between certain degradation parameter and changes in the redox potentials of gold and silver electrodes has been used as a simple yet accurate method for the evaluation of fish freshness. This ''tongue-like'' system that uses the potentiometric signal of unselective gold and silver metallic electrodes has proved to be dependent on the storage time of minced fish and fillets of sea bream. A remarkably good correlation of the potential of the gold and silver electrodes with certain degradation indices, such as changes in pH, microbial counts, nucleoside concentrations, has been found. Of especial interest is the linear correlation between the electrode response and parameters such as the K_1 index that indicates the simultaneous evolution of certain metabolites (inosine 5′-phosphate, inosine and hypoxanthine). The potentiometric method we report herein is fast, low-cost and can be non-destructive and we suggest that it might be applied for *in situ* and *at site* fish freshness monitoring in a wide range of situations. Moreover, this method can be a complement to classical sensory analysis for freshness evaluation and an alternative to other high-cost and time-consuming methodologies. Further studies of repeatability, reliability and accuracy, working with other fish species and storage conditions, will be carried out in due course.

Acknowledgements

We wish to thank the Polytechnic University of Valencia for financial support through the interdisciplinary projects call, the Generalitat Valenciana, and the Ministerio de Educación y Ciencia (project CTQ2006-15456-C04-01) for support. This work was partially carried out under the Associated Unit framework between IIAD (UPV) and IATA (CSIC).

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