БІОСЕНСОРИ

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BIOSENSORS

ELECTROCHEMICAL TONGUE FOR FISH FRESHNESS EVALUATION

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Summary

The fish products are sources of high biological value proteins, of several salts, vitamins so being a valid alternative way to animal foods; fish is rich of essential polynsatured fat acids as preventing factors of some diseases as ateriosclerosis and heart ache.

The aim of research was to yield a new mean for the fish freshness monitoring basing on the modification suffered by fish after his death.

As monitored signals of the proposed "electrochemical tongue" we assumed common index as such as hypoxanthine or phenols concentration, both measured using proper biosensors and other ones such as redox potential, pH, and the antioxidant capacity measured by an enzymatic amperometric biosensor or by cyclic voltammetry. Considering the simplicity of the proposed analysis, the handiness and portability of the instrumental tools used for the electrochemical tongue so permitting in situ determinations and overall the reliability of the system, verified by the comparison with other traditional methods, it seems reasonable to assign a future to the present proposal.

All the signals have allowed us to define the safety ranges and the limit values both by simple analysis and by low cost sensors.

Keywords: fish; freshness; biosensors; electrochemical tongue.

Introduction

The consumption of fresh fish in Italy is considered essential for a correct feeding due to a specific difference between fish products and land animal meat or eggs lies in the lipidic component: fishes are rich of polyunsaturated fatty acids (phospholipids belonging to the metabolic series of linoleic acid) that are essential in human food as fundamental in the prevention of atherosclerosis and in general of cardiovascular diseases [1]. It is consequently of decisive importance both to be able to recognise fresh fish and to ensure its preservation. One of the most frequent practical problems involved is how to evaluate the freshness of this foodstuff. Only during last years some rules about this problem were promulgated [Italian law D.L. 531 (1993)]. Fish transformation processes lead to deterioration of the

product owing to alteration of its organoleptic characteristics (odour, colour, taste, consistency), loss of nutritive value (destruction of vitamins, carbohydrates, proteins) and production of toxic substances. Furthermore, catabolic processes of autolytic and bacterial origin also occur [1]. The former are related to the formation of hypoxanthine, a precursor of uric acid, starting from ATP, while the most typical product of bacterial metabolism is trimethylamine (together with cadaverine, putrescine, tyramine, tryptamine, histamine — the latter three being toxic amines).

The present work describes the development of methods that, when used together, could represent an "electrochemical tongue" able to determine different physico-chemical characteristics such as redox potential, pH and antioxidant capacity, the latter being reversely related also to the production of radicals. For these indexes safety ranges and/or evaluation indexes were identified that could be used, together with more classical indexes, such as hypoxanthine and phenols concentration, to estimate fish freshness. The whole procedure involves using an array of simple, rapid, reliable sensors also capable of being used "in situ".

Experimental

Reagents and Chemicals

Xanthine (2,6-dihydroxy purine) sodium salt, ethylenediamine tetracetic acid (EDTA), superoxide dismutase 4980 U mg^{-1}, dialysis membrane (art. D-9777), *N,N*-dimethyl-p-phenylenediamine dihydrochloride (DMPD), tyrosinase 6050 U mg^{-1} formic acid and β-phycoerythrin supplied by Sigma (Milan). Xanthine oxidase 0.39 U mg^{-1}, cellulose acetate and hypoxanthine supplied by Fluka AG, Buchs (Switzerland). 2,2'-Azobis(2-amidinopropan)dihydrochloride (ABAP), supplied by Waco Chem. (Richmond, VA, USA). Polyvinylacetate, acid-2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman (Trolox), cellulose triacetate, supplied by Aldrich (Germany). Teflon membrane.

Apparatus

Crison pH meter mod. GLP 22; Amel polarograph mod.433-A analyser, mod. 7.0 provided with printer; UV-VIS spectrophotometer Perkin- Elmer, mod. lambda 5, supplied with printer; spectrofluorimeter Perkin-Elmer, mod. LS-5, provided with Perkin-Elmer recorder, mod. 561; elecrode mod. 4000-1 by Universal Sensor Inc. New Orleans, LA, USA, coupled with an Amel potentiostat mod. 551, connected to an Amel differential electrometer, mod. 631 and to an Amel analogical recorder, mod. 868; Ultra-Turrax homogenizer mod. T8 by Ika Labortechnik.

Methods

Sample Preparation

1g of fresh fish pulp sample to be analysed (mullet), previously boned and skinned, was homogenized in 6.0 mL of phosphate buffer at pH 7.5 in a homogenizer at 10000 rpm for 5 min; the homogenate was centrifuged at 3500 rpm for 30 min. Amperometric analysis by enzymatic biosensors was performed on the centrifugate. For the purpose of potentiometric (pH) measures and voltammetric measures, 1.0 g of finely minced sample pulp in 50 mL of distilled water was magnetically stirred for two hours and then left to stand for one hour before being centrifuged at 3500 rpm for 30 min. For the purpose of voltammetric analysis, a supporting electrolyte, KCl 1.0 mol L^{-1}, was added to the supernatant in a ratio of 9 to 1 by volume, respectively (best observed experimental conditions).

The sample was allowed to break down at room temperature regardless of the applied electrochemical method. The time interval over which the chosen food matrix was investigated was set at five days.

Potentiometry

a) Potential measures: fixed aliquots (500 µL) of sample (centrifuged extract) were added to 20 mL of phosphate buffer at pH 7.5 contained in a beaker and under magnetic stirring. The indicator electrode (Pt) and the reference electrode (Hg/ Hg_2Cl_2/KCl_{sat}), both connected to a potentiometer, were immersed in this solution and the potential variation mV recorded as function of time.

b) pH measures: carried out on the same solutions of sample (centrifuged extract).

Cyclic voltammetry [2,3]

10 mL of previously centrifuged sample were added to 10 mL of phosphate buffer at pH 7.5 contained in a thermostated voltammetric cell. Measurement under nitrogen flow involved the use of three electrodes: a glassy carbon indicator electrode, a platinum counter electrode, and calomel reference electrode. To avoid loss of sensitivity the indicator electrode was cleaned after each cycle by abrading the surface with alumina after moistening it with a minimal quantity of distilled water. The voltammogram was recorded over the potential range 1300 ÷ (-200) eV, full scale ± 204,8 мA and scan rate 50 mV/s. For the purpose of the analysis the anodic area of the recorded voltammogram [2,3] was assumed as an indicator of the sample's reducing power, therefore as an index of the antioxidant capacity.

Superoxide dismutase (SOD) biosensor [4,5]

The superoxide radical was determined using a biosensor obtained by coupling a transducer (an amperometric electrode for hydrogen peroxide) with the superoxide dismutase enzyme immobilised in kappa-carrageenan gel. The gel containing the enzyme is sandwiched between a cellulose acetate membrane and a dialysis membrane. The whole assembly is secured to the electrode with an O-ring. A constant potential of +650 mV with respect to an Ag/AgCl/Cl$^-$ cathode is applied to the platinum anode. The dialysis membrane is used to support the gel and to prevent attack by the enzyme. The superoxide radical is produced by the oxidation in aqueous solution of xanthine to uric acid in the presence of the xanthine oxidase enzyme. The disproportion reaction of the superoxide radical, catalysed by the superoxide dismutase immobilised on the electrode, produces oxygen and hydrogen peroxide. The hydrogen peroxide produced is oxidised at the anode, generating an amperometric signal (in nA) that is proportional to the concentration of the superoxide radical present in solution. The addition of a sample possessing antioxidant properties produces a decrease in the signal as, by reacting with the superoxide radical, the concentration of these species in solution is lowered. As a consequence, both released H_2O_2 and intensity of the amperometric current diminished. The electrochemical biosensor was placed in a cell thermostated at 25°C, containing 15.0 mL of phosphate buffer 0.05 mol L^{-1} at pH 7.5 and allowed to stabilise under constant stirring. After addition to the same solution of a fixed quantity of the xanthine oxidase enzyme (1.2 mg), two successive additions of 200 µL of the solution of xanthine 0.01 mol L^{-1} were added, waiting for the signal stabilising between successive additions. The value of the recorded current variations were thus plotted versus the angular coefficient could be calculated.

The value of the anti-oxidant capacity was expressed by the following algorithm:

$$\text{(RAC) "Relative Antioxidant Capacity"} = 1 - (m_c / m_x)$$

m_x = angular coefficient of the straight line obtained by means of successive additions of xanthine

m_c = angular coefficient of the straight line obtained by means of successive additions of xanthine, but in the presence of the sample possessing anti-oxidant properties

as described in detail in previous papers [4,5]

Xanthine oxidase electrochemical biosensor [6]

The hypoxanthine produced during the decomposition of the ATP into uric acid in the chosen food matrix was determined using an electrochemical biosensor constructed by coupling a transducer (a gaseous diffusion amperometric oxygen electrode) with the xanthine oxidase enzyme immobilised in a cellulose triacetate membrane. The latter was sandwiched between a teflon membrane and a dialysis membrane. In order to enhance enzyme stability the membrane containing the xanthine oxidase (12 units in 150 μL of phosphate buffer 0.06 mol L^{-1} at pH 7.8) was maintained at 5°C for 48 hours. The solution inside the oxygen electrode consisted of KCl 0.1 mol L^{-1} at pH 7.8. The electrode had a platinum cathode and an Ag/AgCl/Cl$^-$ anode, between which a constant potential of –650 mV was applied. Hypoxanthine determination was performed by recording the amperometric signal variation, which was correlated with the reduction in dissolved oxygen in solution due to the enzymatic reaction:

$$\text{hypoxanthine} + O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + H_2O_2$$
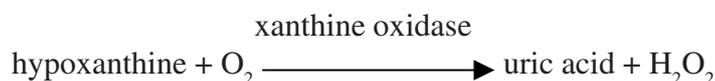
The signal variation due to O_2 consumption is proportional to the concentration of the hypoxanthine that gradually accumulates in the sample as the chain of decomposition reactions has its weakest step precisely in the transformation of the hypoxanthine produced to uric acid. In performing the measurement the electrode was placed in a cell thermostated at 25°C, containing 15.0 mL of phosphate buffer 0.06 mol L^{-1} at pH 7.8 and allowed to stabilise under constant stirring. Successive additions of 200 μL of sample were made and the signal variation recorded after each addition.

Tyrosinase electrochemical biosensor [7]

Phenol content was determined according the following enzymatic reaction:

$$\text{phenol} + O_2 \xrightarrow{\text{tyrosinase}} 1\,,2\text{-benzoquinone} + H_2O$$

The biosensor used was obtained by coupling a Clark electrode, as transducer, with immobilised in kappa-carrageenan gel tyrosinase enzyme. The gel containing the enzyme is sandwiched between a teflon membrane and a dialysis membrane. The internal solution of the oxygen electrode consisted of KCl 0.1 mol L^{-1} and phosphate buffer 0.06 mol L^{-1} at pH 6.6. The electrode comprised a platinum cathode and a Ag/AgCl/Cl$^-$ anode between which a constant potential of -650 mV was applied.

Phenol content was determined by plotting variations in the recorded amperometric signal against the consumption in dissolved oxygen in the solution, due to the enzymatic reaction. Signal variation due to O_2 consumption was proportional to the concentration of the phenol's "pool" contained in the sample. The reaction involved the phenol –OH group. In performing the measurement the biosensor was placed in a cell thermostated at 25°C, containing 15.0 mL of TRIS buffer 0.1 mol L^{-1} at pH 8.5 and allowed to stabilise under constant stirring. 200 μL of the sample were added and the signal variation recorded. Likewise, 200 μL of a standard phenol solution 0.1 mol L^{-1} were added and the signal variation again recorded. Comparison between signal variation due firstly to the addition of the sample and then to that one of the phenol allowed the phenol content of the sample, expressed as mol L^{-1} of phenol, to be determined.

Spectrophotometric and fluorimetric methods for antioxidant capacity measurement comparison

In order to verify the results referring to antioxidant capacity obtained using the superoxide dismutase biosensor, we determined the antioxidant capacity of the food matrix also using two official methods described in the literature. We applied the DMPD+FeCl$_3$ spectrophotometric method [8], according to which at an acid pH and in the presence of a suitable oxidising solution, DMPD (N,N-dimetyl-p-phenilenediamine dihydrocloride) forms a stable coloured cation radical according to the following reaction:

$$DMPD_{(colourless)} + oxidant(Fe^{3+}) + H^+ \longrightarrow DMPD\cdot+_{(purple)}$$
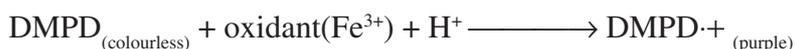
The UV-VIS spectrum of DMPD$^{+\cdot}$ displays peak absorbance at 514 nm. The antioxidant compounds (AOH), which are capable of transferring one hydrogen atom to the DMPD$^{+\cdot}$, produce a decoloration of the solution (which is recorded) proportional to their concentration according to the reaction:

$$DMPD^{\cdot+}_{(purple)} + AOH \longrightarrow DMPD^+_{(colourless)} + AO$$
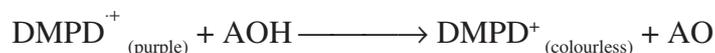
The second method used was ORAC (Oxygen Radical Absorbance Capacity) spectrofluorimetric method [9,10]: in the presence of free radicals or oxidising species, the protein β-phycoerythrin (β–PE) loses more than 90% of its fluorescence within 30 min. The addition of antioxidant species, which react with the free radicals, inhibits the fluorescence of this protein. This inhibition can be correlated with the sample's antioxidant capacity. In our particular case, 2,2-azobis-(2-amidinopropane)dihydrochloride (ABAP) was used to generate peroxide radicals (ROO$^\cdot$).

Results and discussion

The present research aims to provide a contribution to modern food monitoring procedures by identifying several experimental markers that can be used to determine fish freshness and that can easily be detected using electroanalytical or biosensor-based methods. In this connection initially we particularly determined redox potential by means of direct classical potentiometry (Fig.1) and by a platinum electrode, we established that for very fresh sample (at the first day) thus value does not exceed 50 mV so allowing to have a first assessment of freshness.

The results obtained using cyclic voltammetry not only allowed a value to be determined for the anodic area (i.e. the reducing power), which represents the method's response to the variations of the fish freshness within a defined time (this value must have a value of at least 7 cm^2, which corresponds to an equivalent ascorbic acid concentration of 1.9×10^{-3} mol L^{-1}), but also it allowed the confirmation of the results obtained by other electroanalytical methods. The anodic area correlates with both the measurement of overall reducing power, and with the measured antioxidant capacity. Indeed, since it is evident that the reducing power of a substance represents a valid expression of its antioxidant capacity, the anodic area of the voltammogram may be correlated directly with this capacity (Fig.2). Also in this case, the variation in the fish's antioxidant capacity was investigated within five days using both a method based on an SOD electrochemical biosensor [4,5] and by means of cyclic voltammetry [2,3], as well as by using two reference methods described in the literature: a spectrophotometric method (DMPD-FeCl$_3$) [8], specific to lipid matrixes and the other a spectrofluorimetric method (ORAC) [9,10]. Using each of these methods a clear-cut difference in antioxidant capacity was found between the first day and the fifth day of the analysis (Fig.3). All four methods gave a good indication of the freshness of the fish sample investigated and allowed antioxidant capacity to be determined, although

of course the numerical values were found to be different in all four cases. This was because each method used measured different signals and different numerical scales were naturally used to express the antioxidant capacity. A relatively feasible interpretation of this chemical property can be attributed to the development of phenols (typical antioxidants) which are found in increasing amounts in the fish sample with the passing of time (Fig.4). Using the SOD biosensor we obtained a maximum estimated value of RAC of under 0.5, which was taken as an index of freshness. On the other hand, in order to judge a fish as fresh the antioxidant capacity value, expressed as an ORAC value or in TEAC units must not exceed the values of 35 (ммol of Trolox equivalent) and 5.0 (µg of Trolox equivalent), respectively. We therefore determined the maximum value of phenol content above which the fish may no more be considered fresh (3.20×10^{-2} mmol L^{-1}). We also investigated the correlation between these values and the results in terms of antioxidant capacity and pH (Fig.5). Indeed, the results of the tests based on pH measurement are an expression of the simultaneous nature of two processes that take place during the decomposition of fish, i.e. the production of putrefaction amines, in the first instance, hypoxanthine (Fig.6 (a)), which also leads to an increase of pH values, and the increase in phenol concentration, which is believed to produce a relative acidification. Nevertheless it must be concluded that the phenols are able to neutralise only a part of the produced amines as, with the passing of time, pH increases and so the amine-generating putrefactive process seems to be prevailing on the phenol one.
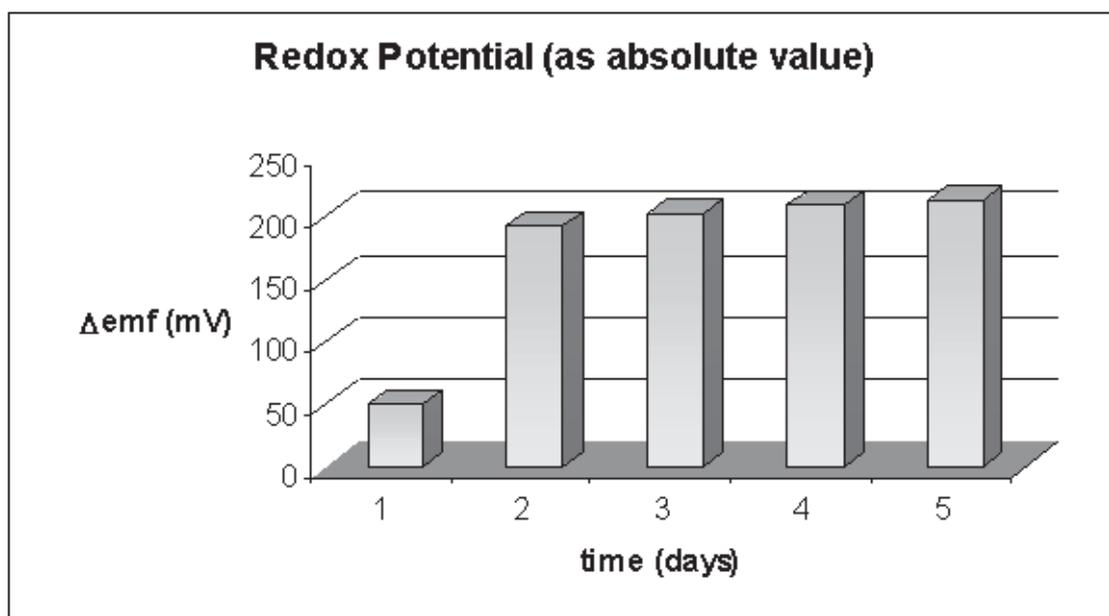

Fig. 1. Behaviour of redox potential recorded during the first five days since the death of fish.

On observing the behaviour of the hypoxantine concentration with the passage of the time, Fig.6 (a), it is concluded that it is not completely agreeing with some other experimental results, as a maximum is present at the 3rd day, probably due to the production of hypoxanthine in the food especially during the first three days. After three days hypoxanthine seems to be degraded to xanthine and to uric acid; however a certain increase of amine concentration seems to occur correspondingly to the fifth day. We have also observed that at the same time also the bacterial concentration increases with henancing oxygen consumption (Fig.6 (b)), particularly evident on the fifth day. So the total behaviour described in Fig.6 (a) seems to be essentially due to a combination of oxygen consumption because of the enzymatic reaction catalysed by xanthine oxidase, reaching its top during third day and of the growing bacteria reaching then top during fifth day. Anyway the behaviour shown in Fig.6 (a) is

enough agreeing with the results obtained by pH values, Fig.5. Lastly Table 1 contains a summary of the numerical values obtained using the different methods, which may be taken as indicators of the freshness of the tested fish samples.
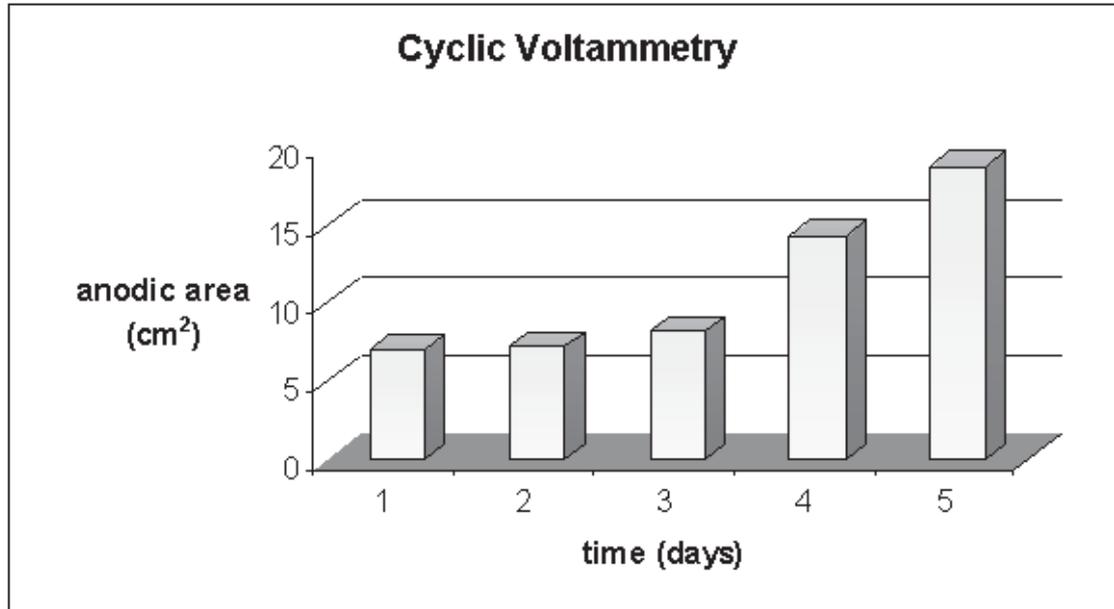

Fig. 2. Behaviour of anodic area recorded during the first five days since the death of fish.
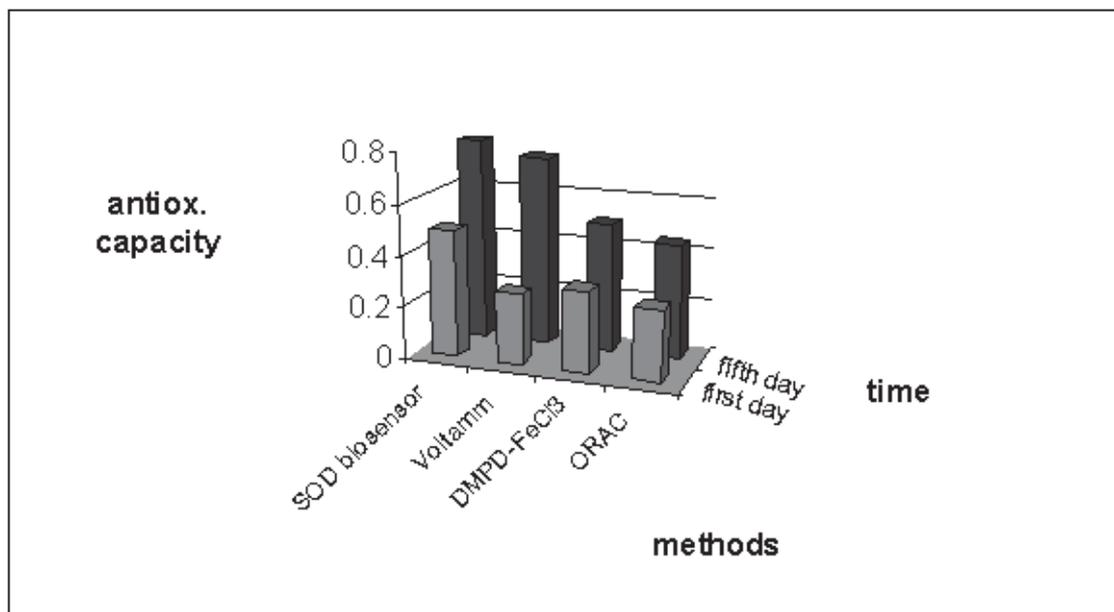

Fig. 3. Comparison of antioxidant capacity at first and fifth day using different electrochemical, photometric and fluorimetric methods. To compare graphically the experimental data, the main values obtained by cyclic voltammetry were divided by 25, those by SOD biosensor assumed as such, those ones by DMPD-FeCl$_3$ divided by 20 and finally those ones by fluorimetric ORAC method divided by 100.
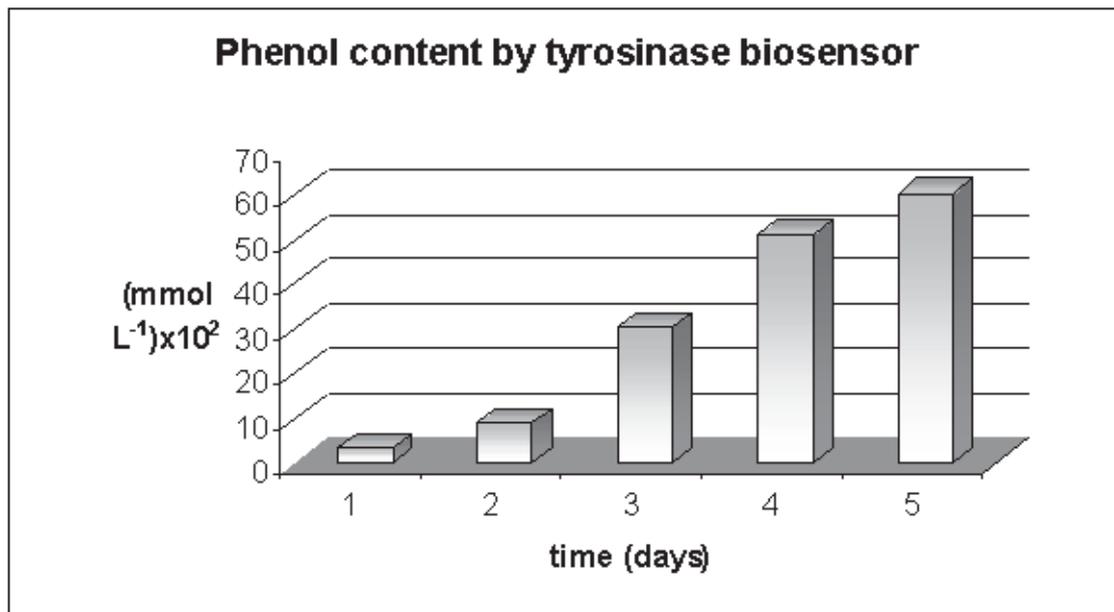
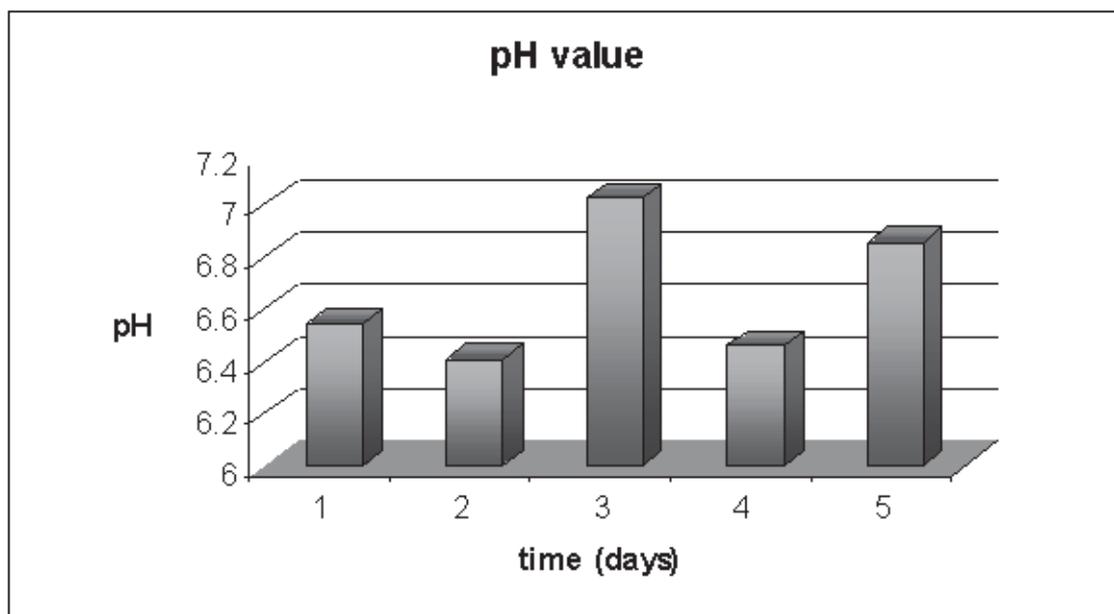Fig. 4. Behaviour of phenol content recorded during the first five days since death.

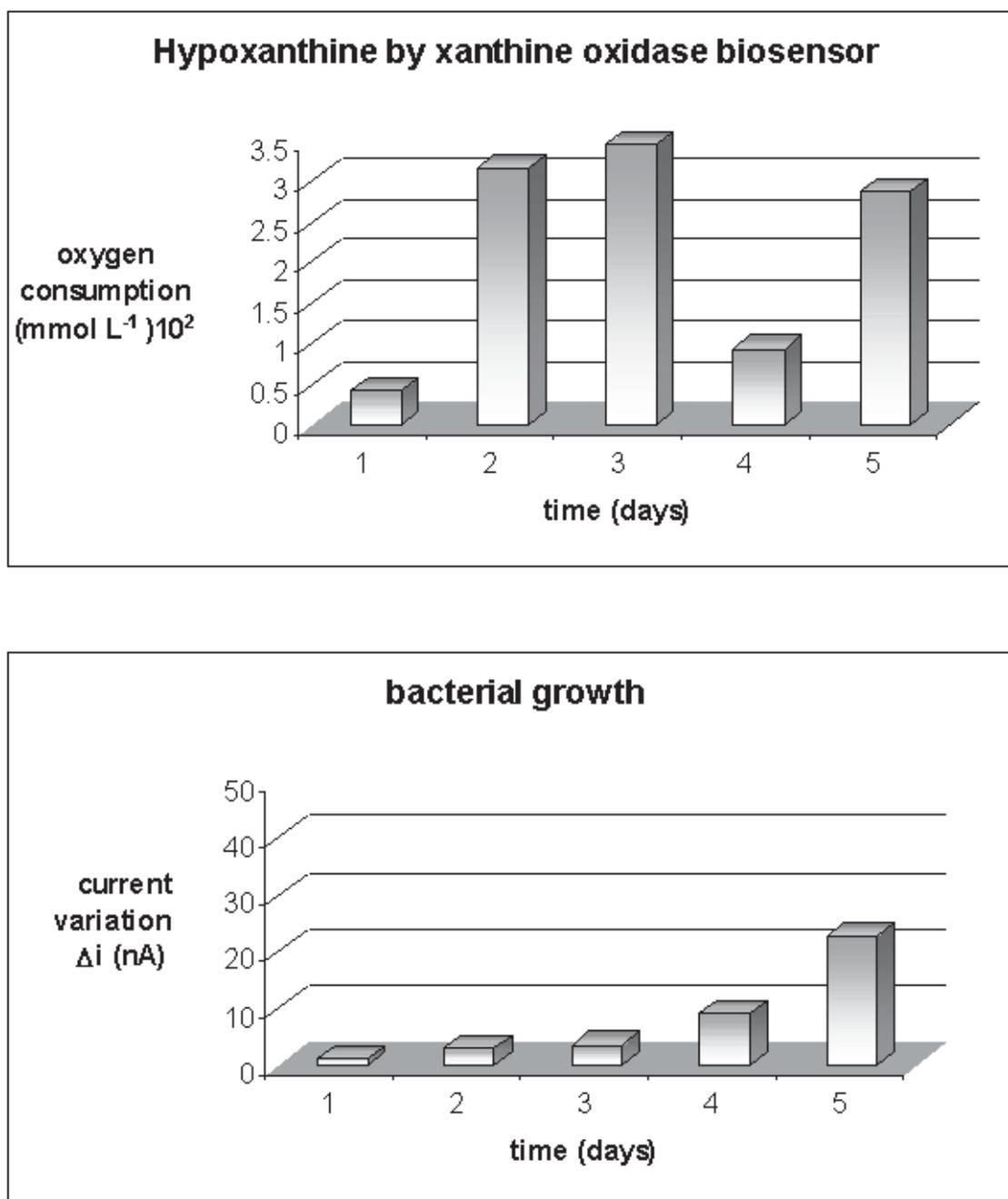Fig. 5. Behaviour of pH values recorded during the first five days since death.

Fig. 6. (a) Trend of hypoxanthine concentration in the analysed sample. Behaviour recorded during the first five days since death. (b) Trend of current variation, due to the increasing oxygen consumption, as the bacterial concentration increase. Behaviour recorded during the first five days since death.

Table 1.
Fish freshness indexes obtained using different electroanalytical methods.

Δf.e.m. (mV)	pH	Anode area (cm^2)	SOD Biosens. (RAC units)	TEAC (µg trolox equiv.)	ORAC (µmol trolox equiv.)	Phenol conc. (mmol L^{-1})	Hypoxanthine conc. (mmol L^{-1})
≤ 50	≤ 6.5	< 7	≤ 0.5	≤ 5.0	≤ 35	≤ 3.2x10^{-2}	≤ 4.2x10^{-3}

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Conclusion

It may be asserted that, considering the simplicity and rapidity of the proposed tests, the low cost of the electrochemical sensors and biosensors used, including the SOD biosensor developed in our laboratory, their easy transportability, which enables them to be used also "in situ", as well as their high reliability, the array of the proposed electrochemical and biosensor tests could be considered as a probe of the electrochemical tongue type for determining fish freshness.

References

1. Giuliano R., Stein M.L., Quaderni di Chimica degli Alimenti, Bulzoni Editore s.r.l., 1975, **5**, 1-18, 75-82 p.
2. Chevion S., Roberts M.A., Chevion M., Free Rad. Biol. Med., 2000, **28**, 860-870 p.
3. Castilho M., Almeida L.E., Tabak M., Mazo L.H., Electrochimica Acta, 2000, **4,** 67-75 p.
4. Campanella L., Favero G., Tomassetti M., Anal.Letters,1999, **32(13)**, 2559-2581 p.
5. Campanella L., Favero G., Persi L., Tomassetti M., J. Pharm. Biomed. Anal., 2000, **23,** 69-76 p.
6. Watanabe E., Ando K., Karube I., Matsuoka H., Suzuki S., Journal of Food Science, 1983, **48**, 496-500 p.
7. Campanella L., Favero G., Sammartino M.P., Tomassetti M., Talanta, 1994, **41**, 1015-1023 p.
8. Fogliano V., Verde V., Randazzo G., Ritieni A., J. Agric. Food. Chem., 1999, **47,** 1035-1040 p.
9. Cao G., Verdon G., Wu C.P., Wang A.H.B., Prior R.L., Clin. Chem., 1995, **41**, 1738-1744 p.
10. Cao G., Alessio H.M., Culter R.G., Free Radical. Biol. Med., 1993, **14**, 303-311 p.