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Determination of hydrogen peroxide in disinfectant solutions using a biosensor with two antagonist enzymes

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Abstract

The development and characterisation of a new biosensor for hydroperoxides is described, which is obtained by combining an oxygen gas diffusion amperometric electrode and two immobilized enzymes (peroxidase and tyrosinase) working in parallel and competing for the same substrate (catechol). The response of the biosensor to several hydroperoxides was investigated (LOD = $0.5 \cdot 10^{-4}$ M for hydrogen peroxide). It was experimentally found that the biosensor is able to respond also to aqueous solutions of ionic peroxides (LOD = $0.2 \cdot 10^{-4}$ M for potassium peroxidisulphate). The biosensor was applied to the determination of the hydrogen peroxide content of pharmaceutical products, i.e. aqueous disinfectant solutions (RSD% $\leqslant 0.5$; recoveries by standard addition method between 96.0 and 98.5%).

Keywords: Enzyme electrode; Hydroperoxide; Disinfectant solutions; Analysis

1. Introduction

The use of a Clark (amperometric gaseous diffusion) electrode as transducer has characterized the development of many of the enzymatic biosensors developed by us in the past for operation in aqueous solvent [1–4], but in some cases also in organic solvent [5,6]. Indeed, the use of such an electrode in biosensor construction ensures several advantages, above all because it is practically free of all interference due to the possible presence of other analytes in solution. The classical amperometric electrode for hydrogen peroxide, which uses a platinum anode polarized at >0.7 V with respect to an Ag/AgCl/Cl^- cathode [7,8] or other types of amperometric electrodes [9,10], also provides a good but comparatively unselective electrode. Since hydrogen peroxide acts as a substrate for the peroxidase enzyme reaction, the enzymatic solution of this enzyme, or the immobilized enzyme, is often used to fabricate amperometric biosensors [11–15] for hydrogen peroxide measurement that are generally slightly more selective than when a simple electrode for H_2O_2 is used. However, oxygen concentration does not vary during the peroxidase

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reaction, so the Clark probe cannot instead be used as a more selective indicator electrode in the construction of a hydrogen peroxide biosensor [16]. Nevertheless, some interesting examples of biosensors using two enzymes have recently been proposed. The enzymes act in parallel and antagonistically, and an amperometric gaseous diffusion oxygen electrode is used as transducer [16]. By using a geometry specific to this biosensor type and two enzymes (peroxidase and tyrosinase) that compete for the same substrate, catechol, it was possible to construct an excellent biosensor for hydrogen peroxide. After the biosensor had been characterized from the electrochemical, enzymatic and analytical point of view, it was used extremely effectively to determine the hydrogen peroxide content of aqueous solutions used as pharmaceutical disinfectants. The analytical results obtained and the comparison with those found by classic titration using potassium permanganate are described in this paper.

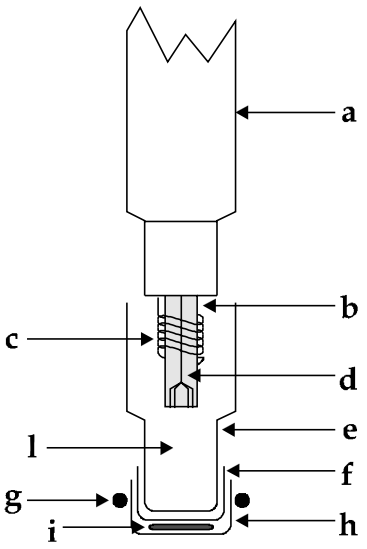

Fig. 1. Biosensor assembly. (a) Gas-diffusion amperometric electrode; (b) dielectric; (c) Ag/AgCl anode; (d) Pt cathode; (e) electrode cap; (f) gas-permeable membrane; (g) rubber O-ring; (h) dialysis membrane; (i) immobilised enzyme; (l) filling solution (phosphate buffer 0.067 M, KCl 0.1 M, pH 6.6).

2. Experimental

2.1. Apparatus

The following apparatus was used in the present research: a 'Biosensor Amperometric Detector' and a 'Model 4000-1' electrode for oxygen measurement, both supplied by Universal Sensor INC (New Orleans) and a model 868 Amel (Milan) recorder. The electrode used was of the gaseous diffusion type, which allows oxygen to be determined amperometrically; it is composed of a platinum cathode and an Ag/AgCl/Cl^- type anode, both immersed in a solution of phosphate buffer (1/15 M) and KCl (0.1 M) at pH 6.6. The internal solution was contained in a cylindrical plastic cap, the lower extremity of which was sealed by a Teflon gas-permeable membrane secured to the cap by an O-ring that prevented the passage of electrolytes and of the solution but allowed that of oxygen. The cap, filled with internal solution, was screwed on to the body of the electrode. A sketch of the electrode and cap is shown in Fig. 1. The Teflon gas-permeable was supplied by Universal Sensor Inc (New Orleans). The D-9777 type dialysis membrane used was supplied by Sigma (St. Louis, MO). The biosensor experiments were carried out at 25 °C in a 15 ml thermostable glass cell supplied by Marbaglass (Rome), connected to a Julabo C thermostat. The solvent used for the tests was kept under constant stirring using a magnetic microstirrer supplied by Velp Scientifica (Italy). Titrations were performed using a 25 ml burette (with $1/10 \pm 0.03$ ml graduations).

2.2. Reagents

Hydrogen peroxide 30% (m/v) from Merck (Darmstadt, Germany); monobasic potassium phosphate, dibasic sodium phosphate, sulfuric acid, 96% (analytical grade), potassium permanganate RPE-ACS, were supplied by Carlo Erba; potassium chloride, κ-carrageenan, tert-butylhydroperoxide aqueous solution (70+30%) (v+v), potassium peroxydisulphate, 3-chloroperbenzoic acid, peracetic acid, magnesium peroxide, hydrogen peroxide urea adduct, magnesium monoper-

oxyphthalate, were all supplied by Fluka; the sodium peroxide was supplied by Sigma. All reagents were 'analytical reagent grade'. The enzymes used-horseradish peroxidase (1510 U/mg, EC 1.11.1.7.) and mushroom tyrosinase (6680 U/mg, EC 1.14.18.1.), were supplied by Sigma.

2.3. Drug samples analyzed

The following is a list of pharmaceutical samples, together with their nominal hydrogen peroxide content as stated by the manufacturers: sample No. 1, aqueous disinfectant solution, H_2O_2 10 vol; sample No. 2, aqueous disinfectant solution, H_2O_2 24 vol; sample No. 3, aqueous disinfectant solution, H_2O_2 10 vol (sample stored without any particular precautions in the laboratory for a long period of time).

3. Methods

3.1. Optimization of the enzyme immobilization method and the ratio between the enzymatic units

In order to optimize the effect on biosensor response of the ratio between the enzymatic units of the two enzymes used, a quantity of peroxidase between 1.8 and 7.1 mg was weighed out, while the quantity of weighed tyrosinase remained constant at 0.7 mg. In this way, three different enzymatic solutions were prepared, all obtained by dissolving the following quantities of enzymes in 25 μl of phosphate buffer (1/15 M pH 6.5): (a) 0.7 mg of tyrosinase, plus 1.8 mg of peroxidase, corresponding to a ratio of 0.5 enzymatic units (peroxidase units/tyrosinase units); (b) 0.7 mg of tyrosinase, plus 3.6 mg of peroxidase, corresponding to a ratio of 1.0 enzymatic units (peroxidase units/tyrosinase units); (c) 0.7 mg of tyrosinase, plus 7.1 mg of peroxidase, corresponding to a ratio of 2.0 enzymatic units (peroxidase units/tyrosinase units).

In order to identify the best enzyme immobilization conditions, three different immobilization methods were tested. First, we investigated simple immobilization in a dialysis membrane [17], where the solutions of weighed enzyme were simply sandwiched between the gas-permeable membrane of the electrode and a dialysis membrane (Fig. 1). Using this method, it is possible to proceed with testing as soon as the biosensor has been assembled.

The second method investigated involved immobilization in a cellulose triacetate membrane [18]. This polymer membrane is prepared using 100 ml of a solution of formic acid/water in the ratio of (9+1) by volume, plus 4 g of cellulose triacetate; the solution is subjected to magnetic stirring for several hours until the polymer has dissolved completely. The polymeric solution obtained is then stratified on a glass support using a suitable stratifier, in order to obtain a membrane $=0.5$ mm thick which is then detached from the support after coagulation in water. Disks, ≈ 3 cm in diameter, are then cut out of it. The disks are then washed in distilled water until the rinsing water is no longer acid.

The third method used was immobilization in κ-carrageenan [19]. A 2% solution by weight of this polysaccharide was prepared by dissolving 0.2 g of it in 10 ml of distilled water; the solution was gently heated and kept under constant stirring for ≈ 15 min. The resulting solution was then poured into a Petri dish and allowed to cool. A gel-like disk was thus obtained having a thickness of ≈ 4–5 mm; 1 cm diameter disks were cut from this and then placed in another dish and allowed to dry.

Each disk (whether of cellulose triacetate or κ-carrageenan) was placed before use in a small container having a diameter of the same order of magnitude as the disk, in which one of the two-enzyme solutions, prepared as described above, was placed; the small container was sealed and stored in a refrigerator at 4 °C overnight.

3.2. Biosensor assembly

The disk containing the immobilized enzymes is positioned at the extremity of the cap of the amperometric gaseous diffusion oxygen electrode, between the gas-permeable membrane and a dialysis membrane; the whole assembly is secured to the electrode cap by means of a rubber O-ring (Fig. 1). After the measurements have been performed, the biosensor cap, with the enzymes

immobilized on it, may be stored in a refrigerator +5 °C, in a damp atmosphere, while, between one measurement and the next, the biosensor is cleaned using the same solvent in which the measurements were performed.

3.3. Biosensor measures (principle of the method)

As already mentioned, the biosensor tested in the present research is based on two oxidation reactions involving the oxidation of the diphenol compound to quinone, which are respectively catalyzed by the enzyme tyrosinase, with consumption of oxygen and by the peroxidase, with consumption of hydroperoxides. On the basis of literature reports [16] describing these two reactions, catechol was selected as common substrate, that is, for both the peroxidase-catalyzed reaction and for the tyrosinase-catalyzed reaction.

3.4. Measurement takes place in two stages

(a) The electrode response is allowed to stabilize, usually for about 1–2 min, (first stationary state), after which a first addition of catechol is made. The addition of this substrate causes a reduction in dissolved O_2 concentration in the solution due to oxidation of the catechol, catalyzed by the tyrosinase (reaction 1). This produces a decrease in the current and then, after ≈ 60 s, when the O_2 consumption rate at the electrode surface becomes equal to the O_2 diffusion rate from the atmosphere to the solution, a new current stationary state occurs (second stationary state).

(b) At this stage, a fixed quantity of a hydroperoxide is added to the solution. After this addition, the catechol is oxidized not only by the O_2 present in solution, but also by the hydroperoxide added, according to the peroxidase-catalyzed reaction (reaction 2). This reaction leads to an increase (i.e. a partial restoration) of the dissolved O_2 concentration in the solution as the hydroperoxide added competes with the O_2 in oxidizing the catechol and so also a partial restoration of the current occurs, until a third stationary state is reached (in ≈ 50 s). The difference in the current value, of the order of several tens of nA, between the last two stationary states, is proportional to the quantity of hydroperoxide added.

$$\text{catechol} + \tfrac{1}{2}O_2 \xrightarrow{\text{tyrosinase}} \text{quinone} + H_2O$$

(reaction 1)

$$\text{catechol} + \tfrac{1}{2}H_2O_2 \xrightarrow{\text{peroxidase}} \text{quinone} + 2H_2O$$

(reaction 2)

3.5. Construction of a calibration curve using the biosensor

According to the scheme described in Section 3.4, the calibration curves were constructed using 8 ml of undecarbonated distilled water, i.e. CO_2 and bicarbonate buffer $6.15 \cdot 10^{-4}$ M at pH 5.6, contained in a 25 ml cell and maintained under constant stirring using a magnetic microstirrer. A special stock solution of hydroperoxide of known titre and one of catechol $6.0 \cdot 10^{-3}$ M were then prepared. Once the signal had stabilized, 1.0 ml of the catechol solution was added. The signal variation was recorded, and the signal then allowed to stabilized again, after which a series of additions of 25 μl of the standard aqueous hydroperoxide test solution were made. After each addition the signal was again allowed to stabilize and the corresponding current variation recorded. At the same time the signal variation was constantly recorded on an analog recorder. The standard solutions of the various hydroperoxides (or peroxides) tested, used to construct the calibration curves case by case, were as follows: hydrogen peroxide $2.7 \cdot 10^{-2}$ M, tert-butylhydroperoxide $3.6 \cdot 10^{-1}$ M, magnesium monoperoxyphthalate $1.1 \cdot 10^{-2}$ M, hydrogen peroxide urea adduct $3.6 \cdot 10^{-2}$ M, 3-chloroperbenzoic acid $2.3 \cdot 10^{-3}$ M, peracetic acid $1.1 \cdot 10^{-2}$ M, sodium peroxide $3.2 \cdot 10^{-2}$ M, magnesium peroxide $2.6 \cdot 10^{-2}$ M and potassium peroxysulphate $1.1 \cdot 10^{-1}$ M.

A typical experimental biosensor response (current versus time), obtained after an initial addition of catechol followed by several subsequent addi-

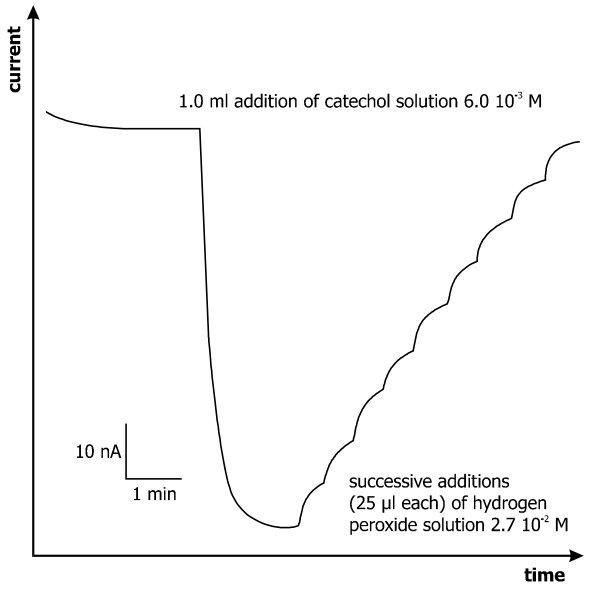

Fig. 2. Biosensor response.

tions of standard hydrogen peroxide solution, is shown in Fig. 2.

3.6. Hydrogen peroxide determination in drug samples

The drug samples analyzed consisted of three different aqueous solutions of hydrogen peroxide for disinfectant purposes contained in plastic bottles. Two of these containers were sealed and opened only just prior to the analysis, while a third container had been opened several months prior to use and then stored in the laboratory without taking any special precautions. A sample of known volume (1.0 ml) was taken from each of the three solutions and then suitably diluted with distilled water before testing. The method used to determine the hydrogen peroxide content of the samples consisted of a direct comparison with a standard solution of known concentration, similar to that of the test sample, both suitably diluted, so that their final solutions lay within the linear range of the method. Also, in this case, 8.0 ml of solvent were used, first adding 1.0 ml of standard catechol solution followed by further 25 µl additions, alternating additions of standard solution with samples of the test solution, each time recording the current variations after each addition. By comparing the latter after the addition of the sample and of standard solution, at least three results were obtained referring to the concentration of sample, which were then averaged.

3.7. Recovery tests on drug samples

Recovery trials using the standard addition method were performed on each of the drug samples tested. In each case the addition of a known hydrogen peroxide standard was made in order to increase the initial concentration as $\approx$2-fold. The samples, before and after the addition of standard, were analyzed using the above-described procedure adopted for individual samples.

3.8. Determination of hydrogen peroxide content of drug solutions by means of titration

The reaction between permanganate and hydrogen peroxide is used [20], adopting a decinormal solution of permanganate, the titre of which has been determined by titration with sodium oxalate. By suitably diluting the drug sample, 25.0 ml of an approximately decinormal aqueous solution of hydrogen peroxide is prepared. From this solution 5.0 ml are taken and placed in a 150 ml flask to which is added 30 ml of distilled water and 10 ml of concentrated sulfuric acid, diluted 1+4 (v+v). Titration is performed at room temperature by introducing the permanganate solution of known titre into the solution to be determined. At the equivalence point, the solution takes on a pale pink color for $\approx$30 s.

4. Results and discussion

The optimization of biosensor sensitivity with reference to the working pH and the quantity of catechol in solution were investigated in previous studies [16]. It was found that the sensitivity of the bienzymatic biosensor response did not vary significantly over the pH range between 5.0 and 7.5, although the peak value was attained with a pH of $\approx$5.5. It was also found that the catechol concentration in the solution had a strong influence

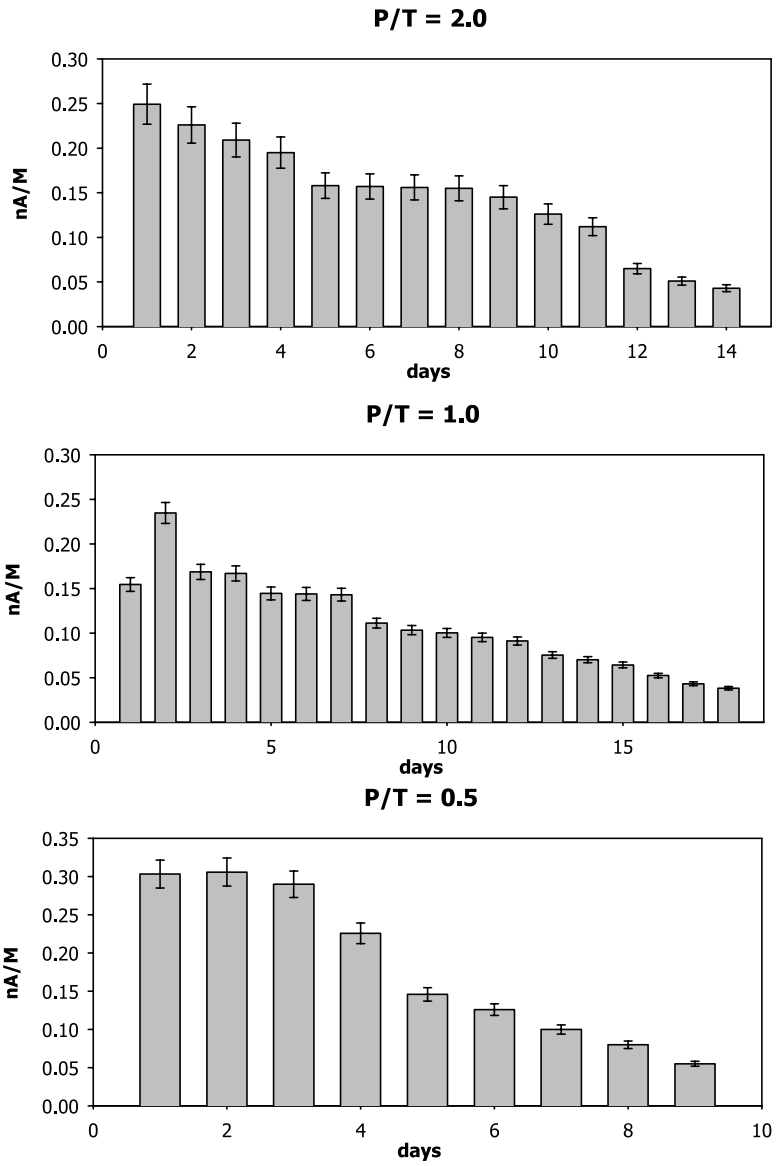

Fig. 3. Variation of sensitivity, as slope of calibration graph, as a function of time, for hydrogen peroxide using different ratios P/T = units of peroxidase/units of tyrosinase (enzymes immobilised in dialysis membrane).

on the sensitivity of the biosensor response as the dissolved O_2 concentration in the solution influences the competition between the two enzymatic reactions. If catechol concentration is too low, the resulting decrease in O_2 concentration in the solution is too small to allow the added hydroperoxides to be determined quantitatively; on the other hand, if catechol concentration is too high, the decrease in O_2 concentration due to the tyrosinase reaction becomes too large, thus decreasing biosensor sensitivity to hydroperoxides. The optimal value of catechol concentration was found to be $6.0 \cdot 10^{-3}$ M [16]. Also the ratio between the enzymatic units of the two enzymes plays a fundamental role in determining biosensor sensitivity, as the hydroperoxides may be deter-

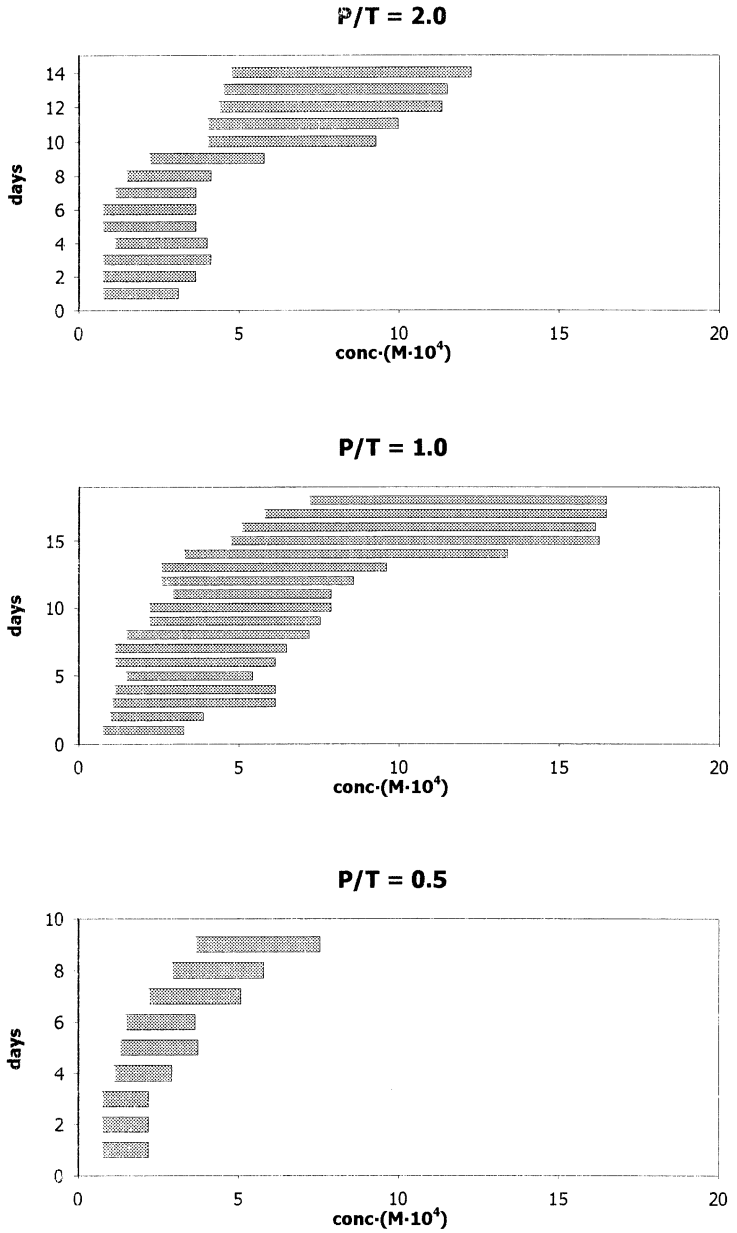

Fig. 4. Variation of linear range of calibration graph, as a function of time, for hydrogen peroxide using different ratios P/T = units of peroxidase/units of tyrosinase (enzymes immobilised in dialysis membrane).

mined on the basis of the competition between the two enzymatic reactions; according to literature reports on the subject [16] it is not however possible to draw any certain conclusions in this regard and so it was decided to conduct an experimental investigation of the effect of the ratio between the two enzymatic units of the two enzymes used on bienzymatic biosensor sensitivity. In these experiments, the biosensor was always assembled in the same way although using differ-

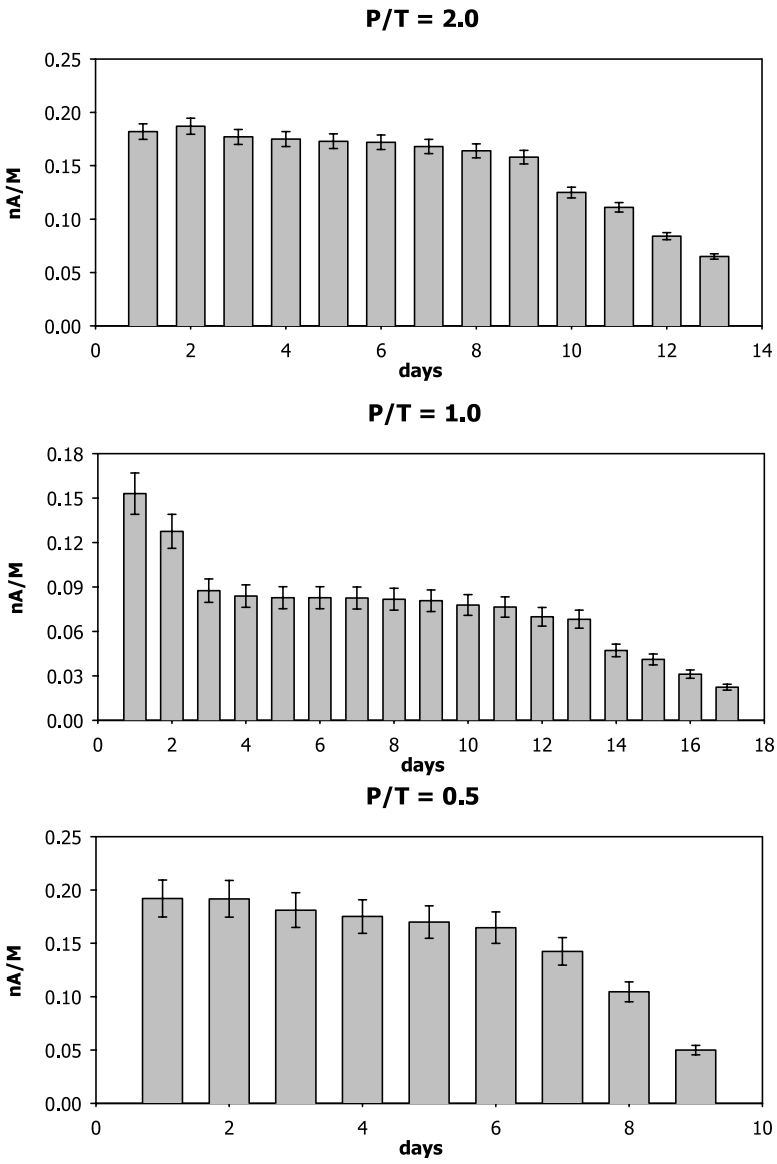

Fig. 5. Variation of sensitivity, as slope of calibration graph, as a function of time, for tert-butylhydroperoxide using different ratios P/T = units of peroxidase/units of tyrosinase (enzymes immobilised in dialysis membrane).

ent ratios between the enzymatic units of the two enzymes used. For this purpose, three different ratios were considered (P/T = units of peroxidase/units of tyrosinase) among the enzymatic units of the two enzymes. As described above, the ratios used were: P/T = 0.5; P/T = 1; P/T = 2. The block diagram in Fig. 3 shows the experimental values of sensitivity, i.e. the slope of the calibration curves, determined throughout the working life of the biosensor, which was assembled with the enzymes simply immobilized in a dialysis membrane and prepared in each case using one of the three different enzymatic ratios described above, following the procedure described in Section 3.8 and using a $2.7 \cdot 10^{-2}$ M standard solution of hydrogen peroxide as substrate and making successive 25 μl

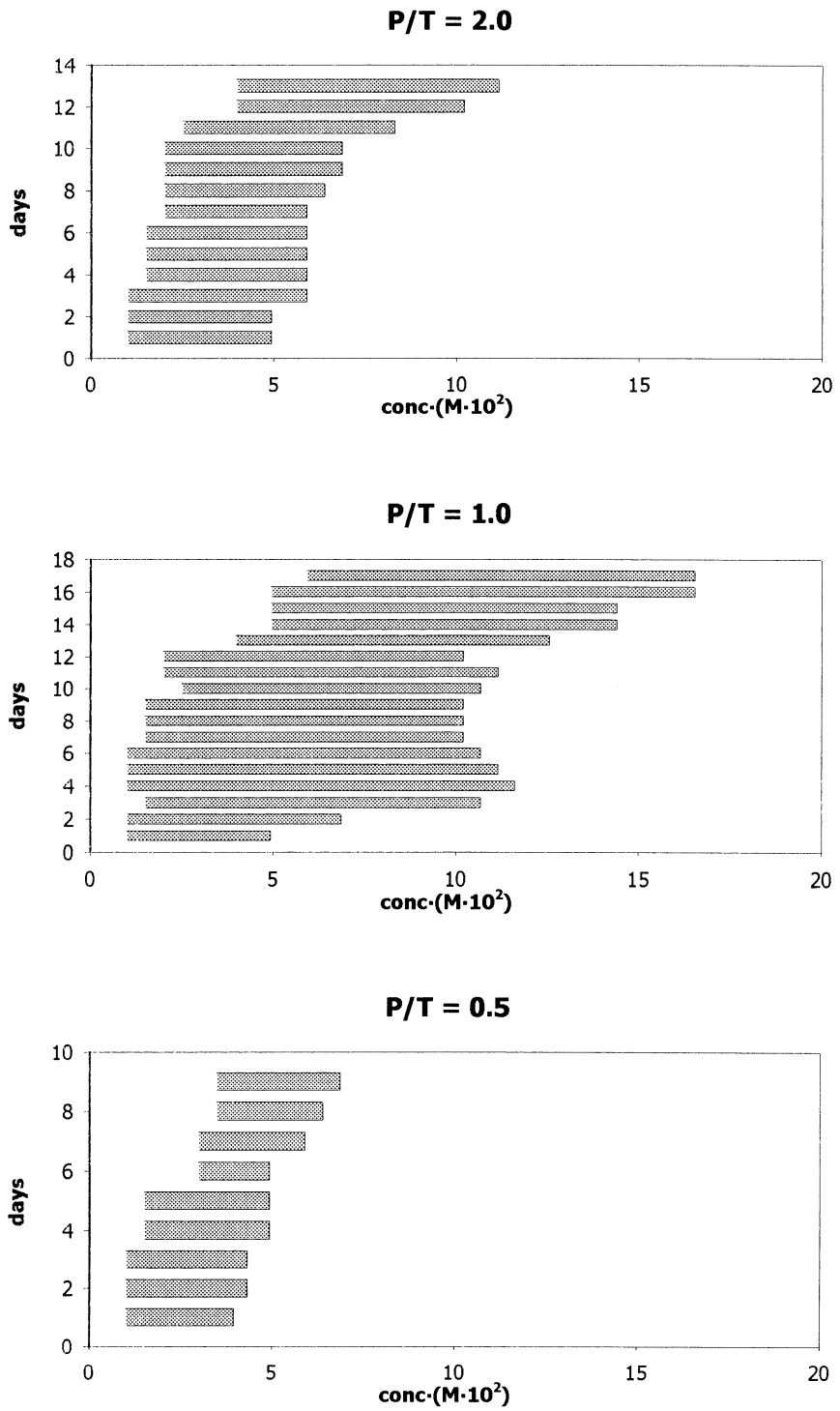

Fig. 6. Variation of linear range of calibration graph, as a function of time, for tert-butylhydroperoxide using different ratios P/T = units of peroxidase/units of tyrosinase (enzymes immobilised in dialysis membrane).

Table 1
Comparison of analytical data for biosensor response to hydrogen peroxide and tert-butylhydroperoxide using different ratios P/T = units of peroxidase/units of tyrosinase (enzymes immobilised in dialysis membrane)

P/T	Equation of calibration curve in the first day (Y = nA; X = M)	Correlation coefficient (r^2)	Linear range (M)	Lifetime (days)	Response time (s)
Data obtained for hydrogen peroxide					
0.5	$Y = 0.30_3$ ($\pm 0.01_7$) $X + 0.09_8$ ($\pm 0.05_3$)	0.9514	$(0.74–2.2_0)\ 10^{-4}$	9	45
1.0	$Y = 0.154$ (± 0.004) $X + 0.019$ (± 0.002)	0.9971	$(0.74–3.2_7)\ 10^{-4}$	18	45
2.0	$Y = 0.24_9$ ($\pm 0.02_2$) $X + 0.05_6$ ($\pm 0.02_0$)	0.9859	$(0.74–3.1_0)\ 10^{-4}$	14	45
Data obtained for tert-butylhydroperoxide					
0.5	$Y = 0.19_2$ ($\pm 0.01_8$) $X + 0.04_3$ ($\pm 0.01_3$)	0.9938	$(1.0_0–3.9_6)\ 10^{-2}$	9	50
1.0	$Y = 0.15_3$ ($\pm 0.01_3$) $X - 0.031$ (± 0.006)	0.9988	$(1.0_0–4.9_3)\ 10^{-2}$	17	50
2.0	$Y = 0.182$ (± 0.003) $X + 0.023$ (± 0.006)	0.9899	$(1.0_0–4.9_3)\ 10^{-2}$	13	50

Table 2
Analytical data for biosensor response to different hydroperoxides (enzymes immobilised in dialysis membrane; P/T = 0.5)

Tested analyte	Equation of calibration curve in the first day (Y = nA; X = M)	Correlation coefficient (r^2)	Linear range (M)
Hydrogen peroxide	$Y = 0.30_3$ ($\pm 0.01_7$) $X + 0.09_8$ ($\pm 0.05_3$)	0.9514	$(0.74–2.2_0)\ 10^{-4}$
Tert-butyl-hydroperoxide	$Y = 0.19_2$ ($\pm 0.01_8$) $X + 0.04_3$ ($\pm 0.05_3$)	0.9938	$(1.0_0–3.9_6)\ 10^{-2}$
Magnesium mono peroxiphtalate	$Y = 0.245$ (± 0.001) $X + 0.008$ (± 0.002)	0.9986	$(0.88–7.3_8)\ 10^{-4}$
Hydrogen peroxide urea adduct	$Y = 0.036$ (± 0.009) $X + 0.02_7$ ($\pm 0.02_6$)	0.9993	$(3.32–29.2_6)\ 10^{-5}$
Peracetic acid	$Y = 0.036$ (± 0.005) $X + 0.017$ (± 0.004)	0.9996	$(3.19–25.0_2)\ 10^{-5}$
3-Cl-perbenzoic acid	$Y = 0.029$ (± 0.004) $X + 0.06_5$ ($\pm 0.04_6$)	0.9824	$(2.61–11.2_9)\ 10^{-5}$

additions of this solution; Fig. 4 instead shows the linear ranges found, while Figs. 5 and 6 show the analogous graphs described above, obtained however using the $3.6 \cdot 10^{-1}$ M standard solution of tert-butylhydroperoxide instead of hydrogen peroxide. In Table 1, it is possible to compare the analytical data referring to biosensor response during the first day of working life for both hydrogen peroxide and tert-butylhydroperoxide, for the three different enzymatic ratios (P/T) considered. The equations of the calibration curves of the linear range, the values of the coefficient of correlation and the linear range in the table are the result of the mean of at least three experimental tests. The table values show that increased biosensor sensitivity towards both hydrogen peroxide and towards tert-butylhydroperoxide is found for the enzymatic ratio P/T = 0.5, even though for this ratio a slightly smaller linear range is found than in the other two cases. Furthermore, the period of use of the biosensor assembled with the enzymatic ratio P/T = 0.5 is 9 days, while it is found to be 17–

Table 3
Analytical data for biosensor response to hydrogen peroxide using three different enzymatic immobilisation methods (P/T = 0.5)

Immobilisation method	Equation of calibration curve in the first day (Y = nA; X = M)	Correlation coefficient (r^2)	Linear range (M)	Lifetime (days)
Dialysis membrane	$Y = 0.30_3$ ($\pm 0.01_7$) $X + 0.09_8$ ($\pm 0.05_3$)	0.9514	$(0.74–2.2_0)\ 10^{-4}$	9
Triacetate cellulose membrane	$Y = 0.20_1$ ($\pm 0.02_0$) $X + 0.11_7$ ($\pm 0.01_5$)	0.9644	$(0.74–2.9_2)\ 10^{-4}$	9
κ-Carrageenan gel membrane	$Y = 0.18_5$ ($\pm 0.04_4$) $X + 0.049$ (± 0.005)	0.9935	$(0.74–4.3_6)\ 10^{-4}$	17

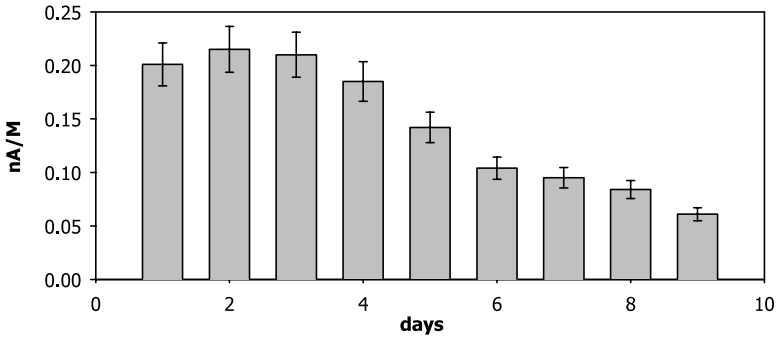

Fig. 7. Variation of sensitivity, as slope of calibration graph, as a function of time, for hydrogen peroxide using a fixed ratio P/T = 0.5 (units of peroxidase/units of tyrosinase), (enzymes immobilised in cellulose triacetate membrane).

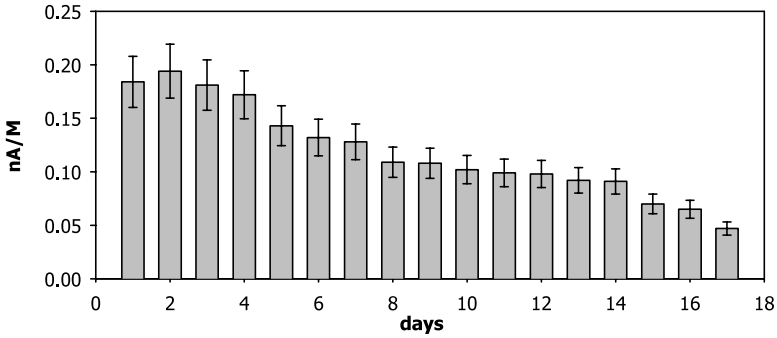

Fig. 8. Variation of sensitivity, as slope of calibration graph, as a function of time, for hydrogen peroxide using a fixed ratio P/T = 0.5 (units of peroxidase/units of tyrosinase), (enzymes immobilised in κ-carrageenan gel membrane).

18 when the ratio is P/T = 1 and 13–14 when the ratio is P/T = 2. The biosensor was subsequently characterized also for other hydroperoxides, also in this case using the enzymatic ratio 0.5, for which biosensor sensitivity was found to be higher. The experimental results are shown in Table 2. As far as biosensor optimization regarding the immobilization method was concerned, calibration curves were constructed using the standard solution of hydrogen peroxide $2.7 \cdot 10^{-2}$ M and with an enzymatic ratio of P/T = 0.5. However, the enzymes were immobilized, not only in a dialysis membrane as already described, but alternatively also in a cellulose triacetate membrane and lastly in a κ-carrageenan membrane. The experimental data obtained (the mean of at least three experimental tests) are compared in Table 3, while the values of the calibration sensitivity, throughout the biosensor's working life, using the two immobilization methods alternative to immobiliza-

Table 4
Analytical data for biosensor response to different peroxides (enzymes immobilised in dialysis membrane; P/T = 0.5)

Tested analyte	Equation of calibration curve in the first day (Y = nA; X = M)	Correlation coefficient (r^2)	Linear range (M)
Sodium peroxide	$Y = 0.29_2\ (\pm 0.08_0)\ X + 0.08_2\ (\pm 0.05_2)$	0.9973	$(0.57–3.6_6)\ 10^{-4}$
Magnesium peroxide	$Y = 0.16_6\ (\pm 0.06_3)\ X + 0.02_6\ (\pm 0.01_2)$	0.9934	$(0.49–4.2_9)\ 10^{-4}$
Potassium peroxidisulphate	$Y = 0.034\ (\pm 0.009)\ X + 0.32_5\ (\pm 0.08_8)$	0.8975	$(1.1_7–4.6_6)\ 10^{-3}$

Table 5
Analysis of different disinfectants aqueous solution containing hydrogen peroxide using biosensor; comparison with data obtained by titration method

Sample No.	Nominal value (w/v)% (a)	Value obtained by titration (w/v)% [RSD%] (b)	Value obtained by biosensor (w/v)% [RSD%] (c)	$[(b-a)/a]$%	$[(c-a)/a]$%	$[(c-b)/b]$%
1	3.07	3.1_2 [0.4]	3.2_4 [4.9]	+1.6	+5.5	+3.8
2	7.38	8.2_5 [0.4]	7.9_5 [5.1]	+11.8	+7.7	−3.6
3	Unknown*	2.3_4 [0.8]	2.2_3 [3.7]	–	–	−4.7

* Original nominal value 3.07 (w/v)%.

tion in a dialysis membrane, are represented in the form of block diagrams in Figs. 7 and 8, respectively. It may be observed that the greatest biosensor sensitivity is obtained using immobilization in a dialysis membrane, while the lowest sensitivity, although still of the same order of magnitude, is obtained using κ-carrageenan immobilization; nevertheless it is precisely this latter type of immobilization that allows the biosensor to be used for a considerably longer period of time than in the other two immobilization methods investigated—as many as 17 days—even using the enzymatic ratio P/T = 0.5. With regard to biosensor assembly, reproducibility may be enhanced by immobilization in cellulose triacetate or in κ-carrageenan membrane: in these cases the reproducibility values, as 'pooled SD', of the enzyme biosensor assembly were estimated as being of the same order of magnitude as the repeatability values of the biosensor response. The precision of biosensor response as 'pooled SD%' and the LOD values for hydrogen peroxide in aqueous solution are 9.5% and $0.5 \cdot 10^{-4}$ M, respectively, while the precision as 'pooled SD%' and the LOD value for tert-butyl hydroperoxide are around 15% and $1.0 \cdot 10^{-3}$ M, respectively. Lastly, also biosensor response towards several peroxides was investigated alternatively using a standard sodium peroxide solution $3.2 \cdot 10^{-2}$ M, a standard magnesium peroxide solution $2.6 \cdot 10^{-2}$ M and a standard potassium peroxydisulphate solution $1.1 \cdot 10^{-1}$ M, adding successive 25 μl aliquots of each of these solutions and proceeding as in the case of the hydroperoxides. The equations of the respective calibration curves over the linear range, the coefficients of correlation and the values of the linear ranges found are shown in Table 4 (mean of at least three experimental tests); the 'pooled SD%' and LOD values for biosensor response to potassium peroxidisulphate are 4.2% and $3.0 \cdot 10^{-4}$ M, respectively.

4.1. Analysis of drug samples

Determination of the hydrogen peroxide contained in samples of pharmaceutical disinfectants was carried out using the biosensor assembled using an enzymatic ratio of P/T = 0.5. Table 5

Table 6
Recovery data for different disinfectants in aqueous solution containing hydrogen peroxide using biosensor

Sample No.	Found H_2O_2 (w/v)% [RSD]	Added H_2O_2 (w/v)%	Total H_2O_2 (w/v)%	Total found H_2O_2 (w/v)% [RSD%]	Recovery (%)
1	7.9_5 [5.1]	8.05	16.0_0	15.7_4 [4.0]	98.4
2	3.2_4 [4.9]	6.15	9.3_9	9.1_3 [4.2]	97.2
3	2.2_3 [3.7]	2.05	4.2_8	4.1_0 [5.8]	95.8

contains all the results obtained from tests carried out using the bienzymatic biosensor on disinfectant solutions. Table 5 also sets out the values for the same real matrixes obtained by classical permanganate volumetric titration, the procedure for which is described in the Section 2; lastly, the Table 5 shows the relative values of precision and correlation between the two methods and with the nominal values. The data contained in Table 5 show that the bienzymatic biosensor method affords good results as far as precision is concerned: for drug samples the RSD% is always $\leqslant$ 5.1. Furthermore, the agreement between the results obtained with the biosensor and the values obtained by volumetric titration (which is taken as reference method) is also very good, always $>95\%$ for all the disinfectant solutions tested. The agreement with the nominal values is good in the case of samples 1 and 2, analyzed immediately after the bottles containing them had been unsealed, while the titre of sample No. 3, conserved in the laboratory without any particular precautions for a long time after opening the bottle, as previously described, was much lower than the nominal value. The confirmation of the fact that the sample had already lost its original titre came from the excellent agreement in the experimental values referring to the hydrogen peroxide content found using both analytical methods (biosensor and titration). Recovery tests carried out using the bienzymatic biosensor and the standard addition method, were carried out on the three drug samples tested. The results obtained, which are also very comforting, are shown in Table 6.

5. Conclusions

The most significant results of the present research are as follows: by coupling an amperometric gaseous diffusion O_2 electrode with two suitable enzymes, it is possible to construct an excellent biosensor for hydroperoxide determination. The two enzymes must not be used to catalyze reactions taking place in series, but must work in parallel and catalyze two different enzymatic reactions that are competing for the same substrate, as in the present research: the two enzymes used in this case peroxidase and tyrosinase compete for the same substrate (catechol). Despite appearances, the resulting analytical method proved to have the required characteristics of ruggedness and robustness. None of the working conditions were found to be 'critical'; for example, small variations in pH or concentration of the catechol added or even in the ratio between the enzymatic units P/T definitively jeopardizes biosensor response, at worst causing relatively small variations.

Enzyme immobilization may be effected in the dialysis membrane, in the cellulose triacetate membrane or in the κ-carrageenan membrane. In the first case, maximum sensitivity is achieved; in the last, the longest biosensor working life is obtained. In comparison with literature reports concerning a biosensor for hydroperoxides of the same type [16], although in the present research the operating parameters were not changed significantly, a much more thorough investigation was made of both the enzymatic ratio (P/T) used and on the various different methods of enzymatic

immobilization, performing a detailed study of the different lifetimes and sensitivity values of the resulting biosensors as a function of the different enzymatic ratios and the different immobilization methods. Above all it was shown that this biosensor can respond not only to hydrogen peroxide but, albeit with variable sensitivity, to different hydroperoxides, such as tert-butylhydroperoxide, peracetic acid, magnesium monoperoxyphthalate, 3-chloroperbenzoic acid, hydrogen peroxide urea adduct. It was also demonstrated that the biosensor can be effectively applied to the analysis of hydrogen peroxide in real matrixes of a pharmaceutical nature. In addition, it was also shown that this biosensor responds also to ionic peroxides, such as sodium peroxide, magnesium peroxide, potassium peroxydisulphate, probably because, in water, these ionic peroxides give rise to the corresponding hydroperoxides [21]. In comparison to biosensors based on the catalase enzyme, previously studied by us [22,23] or by other authors [16], it must be pointed out that, in the case of the catalase biosensor, in the enzymatic reaction of which oxygen is produced, the biosensor response does not remain stable for long once the second stationary state has been reached; it tends to decrease, unless prior deoxygenation of the working solution is performed [16], which instead does not occur in the biosensor described herein; in this case, once the stationary state has been attained, the response is highly stable, which also helps make it highly reproducible.

Finally, using the biosensor certainly makes it easier from both the practical point of view and as regards rapidity and cost-effectiveness, to assay the hydrogen peroxide content of pharmaceutical preparations for disinfectant purposes or of aqueous solutions of hydroperoxides in general.

The precision of these determinations is generally good (RDS% $\leqslant 5\%$), the recoveries obtained using the standard addition method are between ≈ 96 and 98.5%. Lastly, agreement is good also with the classical (permanganate) titration method as the values never differ by $> 5\%$. These results show that the hydroperoxide content of aqueous disinfectant solutions for pharmaceutical use can be analyzed with a precision and accuracy comparable, if not superior, to those obtained using a catalase biosensor [24] and with practically the same selectivity. It may also be claimed that, in recent years, a number of papers have been published by well-known authors, such as Wang et al. [25,26], Turner et al. [27], Avila et al. [28], as well as by the authors of the present article, concerning the possibility of determining hydroperoxides also in hydrophobic pharmaceutical or cosmetic products [24], using peroxidase or catalase biosensors operating in the organic phase. We have already checked that the biosensor described in the present article is also capable, with only a few changes, of being used immersed directly in the organic phase. We are currently completing an investigation devoted specifically to this topic, which will be the subject of a forthcoming publication, including the application of the biosensor to the determination of hydroperoxides contained in hydrophobic cosmetic products and the comparison of the results obtained with those reported by several of the other above-mentioned authors.

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