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Hydrogen peroxide determination in pharmaceutical formulations and cosmetics using a new catalase biosensor[1]

L. Campanella *, R. Roversi, M.P. Sammartino, M. Tomassetti

Department of Chemistry University of Rome 'La Sapienza' p.le A. Moro, 5 00185 Rome, Italy

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Abstract

The possibility of evaluating the content of hydrogen peroxide in several authentic matrices, such as cosmetic and pharmaceutical formulations, was studied. A new catalase biosensor fabricated using an amperometric gas-diffusion oxygen sensor as electrochemical transducer and the catalase enzyme immobilized in kappa-carrageenan gel and capable of operating in both aqueous and non aqueous solvents was developed and tested for this purpose. Creams, emulsions and disinfectant solutions were analysed. To this end, a preliminary check was needed to establish the best conditions to analyse these matrices; the choice of solvent was one of the most important points studied. The solvents considered included dioxane, water-dioxane mixtures, water saturated chloroform and aqueous solutions. The different solubility properties of the matrices analysed were taken into account.

Keywords: Hydrogen peroxide biosensor; Analysis; Cosmetics; Pharmaceutical formulations

1. Introduction

Electrochemical biosensors are modern analytical tools linking classical electrochemistry to the most interesting developments in biotechnology. They now represent a pillar of the recent advances in modern analytical chemistry [1,2]. Electrochemical biosensors used in aqueous solutions (usually buffer solutions) have found numerous applications in the direct analysis of real matrices, also of a pharmaceutical and cosmetic nature [3]. However, many matrices of this type are completely or partially insoluble in aqueous solutions, whereas they generally display a good solubility in certain organic solvents. This of course often limits the potential for performing direct analyses using conventional biosensors.

However, recent research has shown that suitably fabricated enzymatic biosensors can also be used directly also in non aqueous solutions [4–13]. It is consequently possible to envisage using

* Corresponding author. Tel.: +39 6 49913744; fax: +39 6 490375; e-mail: campanella@axrma.uniroma.l.it

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these sensors to determine certain analytes directly in complex matrices that are partially or completely insoluble in aqueous solutions, such as cosmetic creams and emulsions.

Recently J. Wang [14] and, almost at the same time [15], our group, developed a catalase biosensor capable of operating both in aqueous solution and in organic solvents. However, this sensor has so far practically never been used in systematic applications involving real matrices.

The aim of the present research is to fill this gap, that is, to evaluate the possibility of quantitatively determining the hydrogen peroxide contained in certain cosmetic or pharmaceutical matrices using the catalase biosensor developed by us.

In particular, the possibility has been investigated of determining the hydrogen peroxide content of cosmetic and pharmaceutical preparations.

2. Experimental

2.1. Apparatus

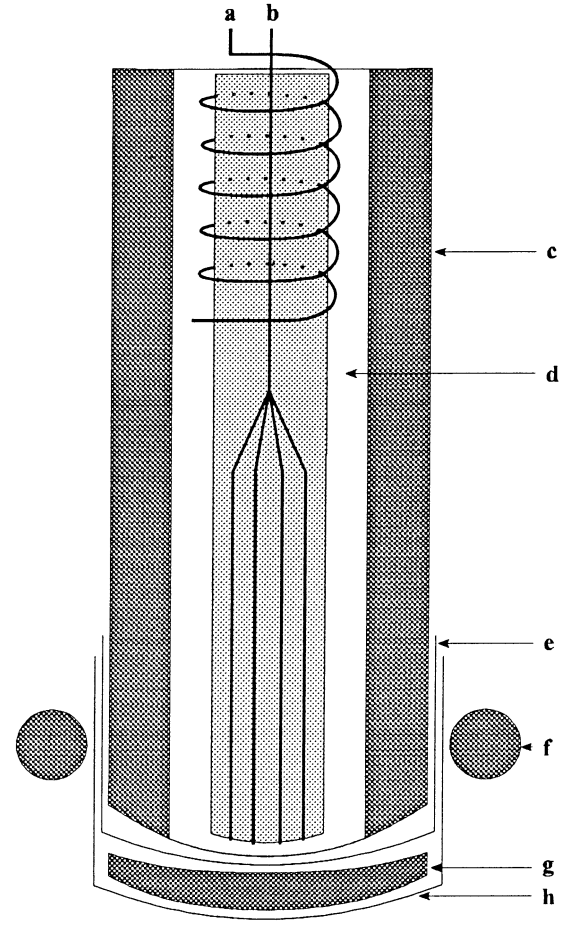
The apparatus used in the present research consisted of an OP-960 Glucose Adapter voltage generator supplied by Radelkis (Budapest), a Mitek-MK 5001 (Taiwan) digital multimeter, an Amel model 868 (Milan) recorder, an oxygen gas diffusion electrode supplied by Universal Sensor (USA). Since the plastic cap of the commercial electrode used was unable to withstand the action of organic solvents, it was replaced by a similar cap of the same size made of teflon. Also the rubber O-ring securing the gas-permeable membrane was replaced by a similar teflon ring.

The electrode and the teflon cap are shown in Fig. 1.

The gas-permeable membrane used, also of teflon, was supplied by Mettler (Germany), while the dialysis membrane used (cat n. D-9777) was supplied by Sigma (USA).

The measures using the biosensor were carried out in a 15 ml thermostattable glass cell supplied by Marbaglass (Italy), connected to a Julabo thermostat (Germany).

The solutions in the measuring cell were kept under constant stirring by means of a magnetic microstirrer supplied by Velp Scientifica (Italy).



$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

Fig. 1. Amperometric gas-diffusion electrode and biosensor assembly: (a) reference electrode (Ag/AgCl); (b) platinum electrode; (c) electrode teflon cap; (d) inner solution (0.1 M KCl); (e) gas-permeable membrane; (f) teflon O-ring; (g) immobilised catalase enzyme; (h) dialysis membrane.

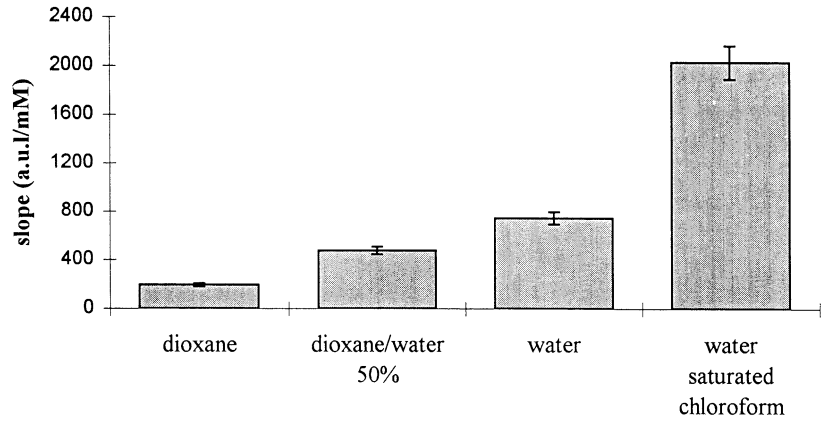


Fig. 2. Trend of the slope of the calibration graph for the hydrogen peroxide, obtained using the catalase biosensor, as a function of the solvent type.

The titrations were performed using a 25 ml burette with 1/10 ml graduations.

2.2. Reagents

The 1,4-dioxane (extra pure) was purchased from Merck (Germany), while the chloroform (for chromatography), the toluene and the chlorobenzene were supplied by Carlo Erba (Italy).

The hydrogen peroxide 30% (w/v) was purchased from Merck (Germany), the potassium monobasic phosphate, sodium bibasic phosphate, the 96% sulfuric acid RPE (for analysis), sodium oxalate (standard for titrimetry), the potassium permanganate RPE-ACS and all the other reagents were supplied by Carlo Erba (Italy). The kappa-carrageenan was purchased from Fluka AG, (Switzerland).

The catalase enzyme, extracted from bovine liver, EC 1.11.1.6, (21000 $U \cdot mg^{-1}$) was supplied by Sigma (St. Louis, MO).

2.3. Cosmetics and pharmaceuticals tested

The commercial samples analysed, all for cosmetic or pharmaceutical purposes, consisted of creams, emulsions or aqueous solutions for disinfecting purposes and contained highly variable amounts of H_2O_2. In some cases the, albeit merely indicative, H_2O_2 content was communicated by the manufacturer; in other cases, no indication of H_2O_2 was given (see Tables 2 and 3).

2.4. Methods

2.4.1. Immobilization of the catalase enzyme in kappa-carrageenan gel

A 2% w/w solution of kappa-carrageenan was prepared by dissolving 0.2 g of polysaccharide in 10 ml of distilled water, heating with caution and maintaining the solution under constant stirring for about 15 min, before pouring the solution obtained on to a Petri dish and allowing it to cool. This produced a jelly-like disk about 4–5 mm thick, from which 1 cm diameter diskettes were obtained using a suitable core extractor. The latter were placed on another dish and allowed to dry at room temperature. A concentrated solution of the catalase enzyme (50 $mg \cdot ml^{-1}$) was prepared in phosphate buffer at pH 7. One of the diskettes thus prepared was placed in a small tube in which 25 μl of the enzymatic solution had previously been placed; the tube was stoppered and left in a refrigerator overnight at 4°C.

2.4.2. Assembly of the biosensor

The diskette containing the immobilized enzyme was extracted from the enzymatic solution and placed between the gas-permeable membrane of the O_2 gas diffusion amperometric electrode and a dialysis membrane; the entire assembly was fixed to the teflon cap of the electrode by means of a teflon O-ring, as shown in Fig. 1.

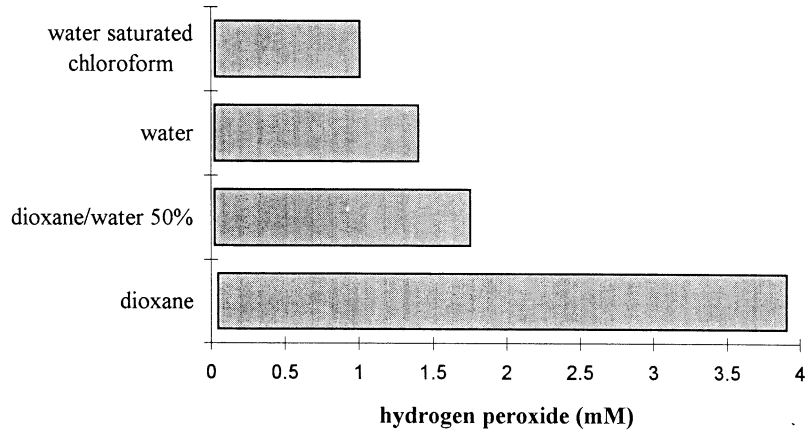


Fig. 3. Trend of the linearity range of the calibration graph for hydrogen peroxide, obtained using the catalase biosensor as a function of the solvent type.

2.4.3. Construction of a calibration graph using the biosensor

The calibration graphs were constructed using 7.0 ml of solvent contained in a 15 ml thermostatted glass cell and maintained under constant stirring using a magnetic stirrer.

The electrode was immersed in the solvent contained in the cell. When the signal became steady the value was read off from the measuring instrument. For each of the solvents used a hydrogen peroxide standard solution was prepared (generally 1.0×10^{-2} M) in the same solvent (or solvent mixture). Once the signal had become steady, a series of about 15–20 small volumes were added (usually 100 μl) of the standard H_2O_2 solution; the corresponding variations in the signal were read on the multimeter after each addition as soon as the signal reached a new steady state (this point was ascertained by simultaneously recording the signal trend using an analog recorder).

All the H_2O_2 standards consisted of solutions of the same solvent as that used to construct the calibration graph; the titre of the standards had been previously checked using permanganate, as described below.

Only in the experiments carried out in water-saturated chloroform was an H_2O_2 standard in dioxane used to perform the successive additions of hydrogen peroxide, using a procedure developed in previous works [15–17].

2.4.4. Hydrogen peroxide determination in cosmetic and pharmaceutical samples using the biosensor

The creams and emulsions were solubilized and diluted in dioxane, while the aqueous solutions were suitably diluted with distilled water. From each sample a known volume (about 1 ml) was taken and suitably diluted.

In the case of cosmetic samples, insoluble in water, the hydrogen peroxide content was measured successively utilising three different solvents: dioxane, 50% (v/v) water-dioxane and water-saturated chloroform; in the case of pharmaceutical samples containing hydrogen peroxide in aqueous solution, the tests were performed using only aqueous solvent.

Also in this case the biosensor was immersed in 7.0 ml of solvent under magnetic stirring, adding generally 100 μl of the sample suitably diluted with the same solvent (or solvent mixture) as that contained in the measurement cell; from the signal variation it was possible to obtain the concentration of the sample using the appropriate, previously constructed, calibration graph.

Recovery tests using the 'standard addition' method were performed using the same procedure and anhydrous dioxane as solvent in order to obtain a complete solubilisation of the samples and good precision and accuracy.

The titrimetric comparison method described below was used both for the determination of each of the samples tested and for the recovery tests.

2.4.5. Hydrogen peroxide determination using titrimetric analysis

The redox titration method in aqueous or non aqueous solution of hydrogen peroxide with a decinormal solution of potassium permanganate [18] (previously titrated using oxalate) was used both to check the titre of the standard H_2O_2 and as a method for comparing the samples tested [15].

Shortly, 5.0 ml of the H_2O_2 solution were placed in a 150 ml beaker together with 30.0 ml of distilled water and 10.0 ml of sulfuric acid 1:4; the standardised permanganate solution was added dropwise at room temperature. At the turning point the solution took on a pink colour which persisted for at least 30 s.

The titration of of hydrogen peroxide in dioxane, requires a blank titration of the same volume of solvent alone to be performed and subtracted. It was thus decided to use a method adopted during previous research carried out in our laboratory which gave excellent results and required less time to perform [15,17]. The only variant involved in this method is that the titration must be performed in the presence also of 15.0 ml of chloroform. The organic phase remains at the bottom of the beaker, separated from the aqueous phase in which the titration takes place, in such a way the aqueous phase gradually becomes depleted in peroxide due to its reaction with the permanganate, the hydrogen peroxide passes from the organic phase into the aqueous phase where it is titrated without any loss [15].

3. Results and discussion

The initial problem was how to identify the solvent (or solvent mixtures) in which creams and emulsions, which accounted for the majority of real matrices, would be soluble and, at the same time, allowed accurate measures to be performed with the biosensor directly immersed in the solvent concerned. In these conditions the biosensor was expected to display a good sensitivity and a comparatively long lifetime. Previous research [12] had in fact shown that the sensitivity and lifetime of enzymatic biosensors are strongly dependent on the type of solvent used [17,19].

First, solubility tests were carried out on sample 1 (emulsion) and sample 2 (cream). These samples were both found to be insoluble in water, in which they formed suspensions. In toluene and in chlorobenzene, two solvents in which previous research had indicated [17] that biosensor functioned satisfactorily, samples 1 and 2 precipitated out; in chloroform, depending on the actual sample being tested, a tendency to form two phases was observed.

Since just last year E. Magner and A.M. Klibanov, in their work on organic peroxides [20], demonstrated that the catalase enzyme retains part of its activity in anhydrous dioxane, an attempt was made to use this solvent. Furthermore, the dioxane was found to be the only solvent among those tested by us in which also the creams and emulsions tested were completely soluble. Nevertheless, after constructing several calibration graphs using the catalase biosensor immersed directly in anhydrous dioxane, it was found that the sensor, although giving a satisfactory response, did not display a very high sensitivity. It was therefore decided to construct a set of calibration graphs also in several water-dioxane mixtures, with a view to checking whether the sensitivity and other analytical parameters characterizing the biosensor could be enhanced without loss of accuracy due to decreased solubility of the matrix analysed, even when real matrices were used.

The following solvent mixtures were tested: 1, 25, 50 and 75% water-dioxane (v/v). Of course, the biosensor was also characterized in 100% aqueous solution.

At least three calibration graphs were constructed for each solvent tested and used to obtain a mean calibration graph. The main analytical data are shown in Table 1, while the histograms in Figs. 2–5 serve to compare the values of sensitivity (defined as the slope of the calibration graph) and the linearity ranges obtained using the various solvents and mixtures.

Table 1
Main analytical data, relative to the calibration graphs of the hydrogen peroxide, obtained in water/dioxane mixtures and in water saturated chloroform using the catalase biosensor

Solvent mixture (% v/v)	Response time (min)	Linearity range (mM)	Minimum detection limit (mM)	Calibration graph (y = a.u.; x = mM)	Correlation coefficient (r)	Precision on standard (pooled SD%)	Inaccuracy on standard (% values)
100% Dioxane (0% water)	4	0.05–3.86	0.03	$y = 193.2(\pm 6.8)x + 6.3(\pm 0.1)$	0.9999	3.6	−8.1 − +4.1
1% Water	4	0.05–3.64	0.03	$y = 214.4(\pm 7.3)x + 7.8(\pm 0.2)$	0.9998	1.4	−3.1 − +1.9
25% Water	4	0.38–2.93	0.02	$y = 247.3(\pm 4.0)x + 25.1(\pm 0.4)$	0.9984	2.4	−9.9 − +6.3
50% Water	4	0.025–1.73	0.01	$y = 475.0(\pm 32.3)x + 21.7(\pm 1.5)$	0.9995	3.1	−5.4 − +1.7
75% Water	4	0.025–1.39	0.01	$y = 505.0(\pm 36.4)x + 2.3(\pm 0.2)$	0.9981	3.0	−10 − +7.5
0% Dioxane (100% water)	4	0.025–1.48	0.01	$y = 744.6(\pm 65.5)x + 49.7(\pm 3.7)$	0.9929	2.6	−12 − +8.3
Water saturated chloroform	2.5	0.03–0.98	0.01	$y = 2026.0(\pm 273.1)x + 187(\pm 23)$	0.9916	18.1	−36 − +54

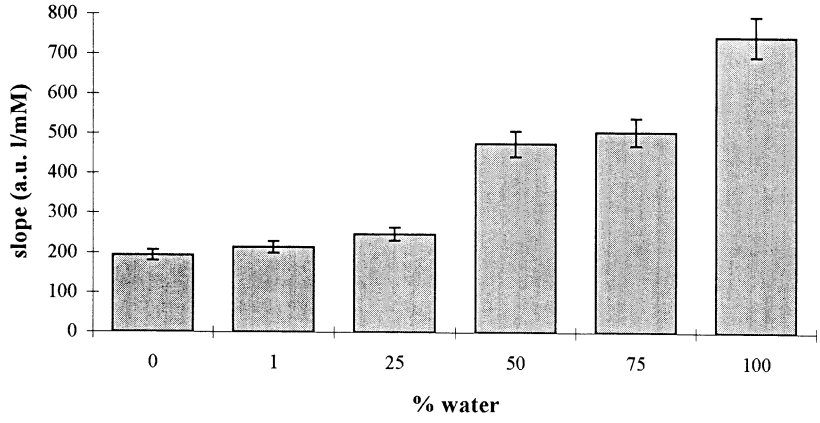


Fig. 4. Trend of the slope of the calibration graph for hydrogen peroxide, obtained using the catalase biosensor, as a function of the water percentage in the water/dioxane mixture.

The sensitivity data trends indicated by the histograms show that, when operating in water, sensor sensitivity is much higher than in anhydrous dioxane, although the linearity range is much greater in dioxane. The 50% (v/v) water-dioxane mixture actually represented a happy compromise as, when this mixture was used, sensor sensitivity proved to be quite high and the linearity range was still quite large (≈ 2 decades).

Conversely, the solubility of the cosmetic samples in this mixture, although lower than in anhydrous dioxane, generally proved sufficient, thanks also to the comparatively low sample concentrations used. Together with pure dioxane this mixture was therefore the one selected for testing all the real samples.

It had been pointed out experimentally in a previous work [15] that water-saturated chloroform is a solvent in which the catalase biosensor gives good results for standard solutions of hydrogen peroxide. It thus seemed advisable to perform the determination of real samples also in water-saturated chloroform. The technique used in this case was the one introduced by Schubert, Turner et al. [21] which had already been successfully used in our laboratory [15] to obtain good calibration graphs by operating in water-saturated chloroform. In practice the cosmetic samples were previously solubilized and appropriately diluted in dioxane, as it was done also for the hydrogen peroxide standard solutions required to obtain the calibration graphs, then small volumes of such solutions (in the order of 100 μl) were used in order to perform normal determinations using the biosensor.

Determination of pharmaceutical samples of disinfectants, which in practice consisted of simple aqueous solutions of hydrogen peroxide, were instead performed in aqueous solutions (phosphate buffer 0.1 M at pH 7 or in simple distilled water).

The results of the tests performed using the catalase biosensor or the volumetric titration to determine hydrogen peroxide content in the cosmetic and pharmaceutical samples are shown in Tables 2 and 3. In the same tables precision and inaccuracy data for both the methods are also reported.

Recovery tests were also carried out on three of the real samples analysed, one for each kind of cosmetic or pharmaceutical form, using the standard addition method. The results obtained by means of both catalase biosensor and volumetric titration, are shown in Table 4.

From the data shown in the Tables 2–4 for the determinations carried out using the catalase biosensor on the eleven cosmetic and pharmaceutical samples considered, it is apparent that the best results were obtained in anhydrous dioxane, as it regards both precision (RSD% ≤ 3.5) and

Table 2
Results obtained using the catalase biosensor in the analysis of the hydrogen peroxide content of cosmetic samples (creams or emulsions) and comparison with data obtained by titrimetric analysis.

Sample no.	Nominal value[a'] (% w/v) (a)	Value found by titration (% w/v) (RSD%) (b)	Value found by the biosensor (% w/v) (c)	RSD%	$\frac{b-a}{a}$%	$\frac{c-a}{a}$%	$\frac{c-b}{b}$%
Creams							
2	6.15	2.31 (3.2)	2.30[b']	1.5	−62.4	−62.6[b']	−0.43[b']
			2.30[c']	4.1		−62.6[c']	−0.43[c']
			2.26[d']	5.2		−63.2[d']	−2.16[d']
4	—	7.26 (0.1)	7.77[b']	1.1	—	—	+7.0[b']
			7.20[c']	3.4			−0.8[c']
			6.13[d']	3.5			−15.6[d']
7	—	3.16 (1.5)	3.15[b']	1.9	—	—	−0.3[b']
			3.01[c']	4.0			−4.7[c']
			2.08[d']	5.1			−34.2[d']
8	—	3.87 (1.5)	4.14[b']	0.9	—	—	+7.0[b']
			3.62[c']	4.4			−6.5[c']
			2.01[d']	5.5			−48.1[d']
Emulsions							
1	12.3	9.58 (1.0)	8.90[b']	3.5	−22.2	−27.7[b']	−7.1[b']
			10.3[c']	7.1		−16.3[c']	+7.5[c']
			4.98[d']	5.2		−59.5[d']	−48.0[d']
3	6.15	4.59 (0.5)	4.59[b']	2.5	−25.4	−25.4[b']	−0.0[b']
			4.81[c']	8.1		−21.8[c']	+4.8[c']
			3.47[d']	4.2		−43.6[d']	−24.4[d']
9	12.3	10.1 (0.9)	10.0[b']	3.3	−17.6	−18.7[b']	−1.28[b']
			9.97[c']	7.5		−18.9[c']	−1.58[c']
			6.35[d']	6.2		−48.4[d']	−37.3[d']
10	6.15	4.93 (1.1)	4.88[b']	2.1	−19.8	−20.6[b']	−1.0[b']
			4.63[c']	6.7		−24.7[c']	−6.1[c']
			2.30[d']	5.4		−62.6[d']	−53.7[d']

[a'] Value (often merely indicative) furnished by the manufacturer, [b'] in anhydrous dioxane; [c'] in water/dioxane (50% v/v); [d'] in water saturated chloroform.

Table 3
Results obtained by the catalase biosensor in the analysis of the hydrogen peroxide content of pharmaceutical samples (disinfectant aqueous solutions) and comparison with data obtained by titrimetric analysis

Sample no.	Nominal value[a] (% w/v) (a)	Value found by titration (% w/v) (RSD%) (b)	Value found by the biosensor (% w/v) (RSD%) (c)	$\frac{b-a}{a}\%$	$\frac{c-a}{a}\%$	$\frac{c-b}{b}\%$
5	3.0	2.74 (3.0)	2.65 (6.7)	−8.7	−11.7	−3.3
6	7.1	6.66 (3.3)	6.33 (6.5)	−6.2	−10.8	−4.9
7	3.0	2.91 (2.9)	2.73 (6.5)	−3.0	−9.0	−6.2

[a] Value furnished by the manufacturer

Table 4
Recovery of hydrogen peroxide in cosmetic and pharmaceutical samples, by the standard addition method, obtained using both the catalase biosensor and the titrimetric method.

Sample no. and cosmetic or pharmaceutical form	Found H_2O_2 (% w/v) (RSD%)		Added H_2O_2 (% w/v)	Total value found by the biosensor (% w/v) (RSD%)	Recovery % obtained by the biosensor	Total value found by the titrimetric method (% w/v) (RSD%)	Recovery % obtained by the titrimetric method
	By the biosensor	By the titrimetric method					
3 (Emulsion)	4.59 (2.5)	4.59 (0.5)	2.55	6.98 (1.8)	97.8	7.12 (3.8)	99.7
	4.59 (2.5)	4.59 (0.5)	5.14	9.22 (1.9)	94.8	9.73 (1.9)	100.0
4 (Cream)	7.94 (1.1)	7.26 (0.1)	2.61	10.6 (2.5)	100.9	8.97 (2.5)	100.0
	7.94 (1.1)	7.26 (0.1)	11.2	18.5 (2.6)	96.9	17.9 (2.6)	96.8
11 (Disinfectant aqueous solution)	2.82 (1.5)	2.91 (2.9)	1.52	4.28 (10.5)	98.6	4.36 (3.0)	98.4
	2.82 (1.5)	2.91 (2.9)	3.00	5.46 (14.3)	93.8	5.81 (1.9)	98.3

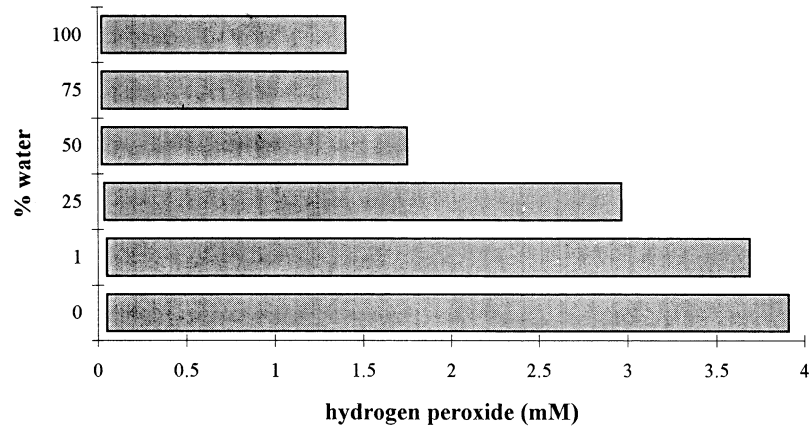


Fig. 5. Trend of the linearity range of the calibration graph for the hydrogen peroxide, obtained using the catalase biosensor, in dioxane/water mixtures, as a function of the water percentage.

inaccuracy ($-7.1 \div +7.0\%$), the latter being evaluated by taking as the true value the one obtained via titration, that is, by taking the titrimetric method as reference method. Also in the 50% water-dioxane mixture and in aqueous solution, the data referring to precision (respectively RSD% ≤ 8.1 and ≤ 6.7) and inaccuracy (respectively $-6.5 \div +7.5\%$ and $-6.2 \div -3.3\%$) were found to be reasonably good; on the contrary in water-saturated chloroform the results were only rarely sufficiently accurate ($-53.7 \div -2.2\%$), while the precision was found to be good also in this case (RSD% ≤ 6.2).

The precision of the value obtained by means of volumetric titrations was of about the same order (RSD% ≤ 3.3) as that found using the biosensor with anhydrous dioxane as solvent. Lastly, also the accuracy evaluated by means of the standard addition method results satisfactory, or at least acceptable: between about 94 and 101.0% using the biosensor and between about 97 and 100% using the titrimetric analysis (Table 4).

In conclusion, a good correlation was found between the data obtained using the volumetric titration and the biosensor and two methods displayed a precision of practically the same order of magnitude; nevertheless, determination using the biosensor was simpler, faster, easier to be automated and therefore more satisfactory also from the economic point of view.

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