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Determination of compounds with anticholinesterase activity in commercial drugs by a new enzyme sensor*

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Abstract: A suitable enzyme sensor for the analysis of anticholinesterase compounds of pharmaceutical interest is described. It is based on the competitive inhibiting properties of these compounds on the enzyme butyrylcholinesterase and it is constituted by a hydrogen peroxide amperometric electrode modified by a superimposed Nylon membrane containing two chemically immobilized biological mediators (butyrylcholinesterase and choline oxidase). Some applications to the analysis of several pharmaceutical forms containing different compounds showing anticholinesterase activity are also reported and evaluated.

Keywords: *Biosensor; anticholinesterase drugs; analysis.*

Introduction

An enzyme inhibition sensor has been developed [1], that is able to determine different compounds showing anticholinesterase activity. The enzyme sensor used was obtained by immobilizing two enzymes (choline oxidase and butytylcholinesterase) on a functionalysed Nylon membrane. The membrane was then coupled with an amperometric electrode, selective to hydrogen peroxide. A solution of butyrylcholine in glycine buffer acted as substrate. The first study investigated the response of the biosensor to several organophosphorus compounds [1, 2].

In this paper the possible application of this new biosensor to the analysis of standard solutions of important compounds with anticholinesterase activity, such as physostigmine, neostigmine, pyridostigmine and edrophonium has been studied. The possibility of determining the same compounds in commercial pharmaceutical preparations is also considered.

Experimental

Chemicals

Choline oxidase (EC 1.1.3.17) from Alcaligenes, 10 U mg^{-1}, butyrylcholinesterase (EC 3.1.1.8, from Horse Serum, 275 U mg^{-1}), butyrylcholine and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide were supplied by Sigma (St Louis, MO, USA). The Pall Biodyne membranes (Nylon 6.6, porosity 0.45 μm) with carboxyl groups on the surface, were obtained from Pall Biodyne s.r.l. (Milan, Italy). Physostigmine, neostigmine, pyridostigmine and edrophonium were supplied by Roche (Milan, Italy). Choline chloride, glycine and other chemicals, of the highest purity were obtained from Farmitalia-Carlo Erba (Milan, Italy). Working solutions of choline and butyrylcholine were prepared by suitably diluting the standards.

Apparatus

Enzymic-amperometric measurements were carried out in a 25 ml glass cell, thermostatted at 25°C, under stirring conditions. The model OP-9439-S hydrogen peroxide electrode used and a OP-970 glucose adapter (Radelkis, Hungary) were supplied by Universal Sensors (USA) and were coupled with a Mitek MK 5001 multimeter and an RC61 radiometer servograph recorder.

Samples

Six different commercial pharmaceutical preparations were analysed: two containing neostigmine, two pyridostigmine and one edrophonium, commercially sold as ampoules or tablets. The last drug was a galenic preparation containing physostigmine. The per cent

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Table 1
Composition of the examined drugs*

Drug No.	Pharmaceutical forms	Composition	Nominal value (as % by wt)
1	Ampoules	Neostigmine methylsulphate	0.05
		Sodium chloride	0.84
		Water	99.12
2	Tablets	Neostigmine bromide	6.52
		Lactosium	65.22
		Magnesium stearate	0.11
		Talcum powder	2.39
		Starch	25.76
3	Ampoules	Pyridostigmine bromide	0.10
		Sodium chloride	0.85
		Sodium acetate	0.16
		Water	98.89
4	Tablets	Pyridostigmine bromide	17.14
		Magnesium stearate	0.14
		Starch	52.43
		Talcum powder	2.00
		Lactosium	13.71
		Silica	14.57
5	Syrup	Physostigmine salicylatum	0.10
		Sucrose	20.60
		Water	79.90
6	Ampoules	Edrophonium chloride	1.00
		Aqueous citrate buffer	99.00

*Drug No. 5 is a galenic preparation.

composition of drugs is shown in Table 1. The clinical use of these drugs is in the diagnosis and treatment of myasthenia gravis and for the reversal of the effects of tubocurarine and other non-depolarizing muscle relaxants.

The analysed drugs were submitted to different pretreatments, depending on the commercially available form. The galenic solution was diluted 10 times before analysis; for drugs in the form of ampoules, five ampoules were opened and their contents carefully homogenized by mixing in a small beaker. For solid drugs in tablet form, five tablets were powdered in a mortar. A quantity of the drug (solid or liquid) was weighed in order to obtain 50 ml of solution at a concentration of about 0.4 mM and dissolved in water. If some turbidity was observed, the samples were centrifuged and filtered before analysis.

Enzyme immobilization and assembly of the sensor

Both the enzymes (choline oxidase and butyrylcholinesterase) were immobilized on a Nylon Biodyne membrane, with carboxyl groups on the surface, using classical methods reported in the literature [3] and described in a previous paper [2]. Briefly, 250 U of lyophilized butyrylcholinesterase and 30 U of lyophilized choline oxidase were stratified on a Nylon membrane (0.6 cm diameter) pretreated as follows: a membrane disk was soaked, under stirring, in 0.5 M phosphate buffer (pH 4.8); an aliquot of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide was gradually added to the buffer solution to obtain a final concentration of 0.1 M. Then the solution containing the Nylon membrane was left under stirring at room temperature for 40 min. The membrane was then washed with buffer solution at pH 7.0 and, on the damp membrane, two enzymes were stratified as described above. Finally, the membrane was left in a humid atmosphere, at +4°C, for 24 h.

The membrane thus obtained was then rapidly washed with glycine buffer 0.1 M (pH 8.0) and superimposed on the head of the hydrogen peroxide electrode and covered with a high mesh Nylon net. Finally, membrane and net were fixed to the electrode by means of a rubber O-ring. The immobilized enzyme-specific activities of choline oxidase and butyrylcholinesterase, measured by the same methods as reported in a previous paper [4], were respectively, 1.3 and 0.5 $\mu m/(min \times cm^2)$ immediately after preparation. After a short stabilization time, about 50–70% of these original activity values is maintained even under working conditions.

Method

The method is based on the following reactions; the analyte serves as an inhibitor for reaction (1):

$$\text{butyrylcholine} \xrightarrow{\text{butyrylcholinesterase}} \text{choline} + \text{butyric acid}, \quad (1)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{choline oxidase}} \text{betaine} + 2H_2O_2, \quad (2)$$

$$H_2O_2 \xrightarrow[+0.6\ V]{\text{Pt/anode}} O_2 + 2e^- + 2H^+, \quad (3)$$

as stated above, both the enzymes are immobilized on a Nylon membrane covering a hydrogen peroxide amperometric electrode. The enzyme electrode is stabilized in 15 ml of glycine buffer solution 0.1 M (pH 8) at 25°C. A suitable amount of substrate (butyrylcholine) is then added, so that the final concentration of butyrylcholine is 2×10^{-4} M. An increase in hydrogen peroxide is immediately observed and the steady-state condition is reached after about 2 min. At this stage the compound with anticholinesterase activity is added and a decrease in hydrogen peroxide in solution is observed. This decrease, measured under new steady-state conditions (after about 3 min), is proportional to the final inhibitor concentration in the buffer solution. This procedure and the sensor response are schematically described in Fig. 1. By a suitable calibration plot, the unknown concentration can be determined. After each measurement, the sensor is renewed by immersion in a stirred butyrylcholine solution for about 3 min.

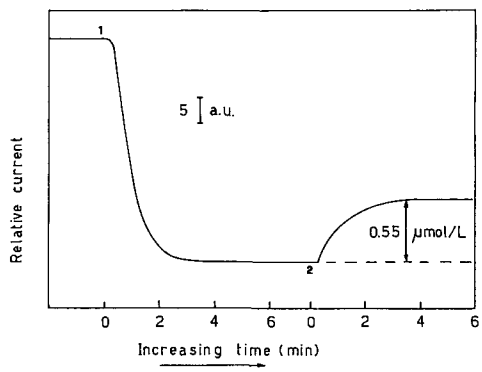

Figure 1
Physostigmine determination. Response of the inhibition enzyme sensor, with choline oxidase and butyrylcholinesterase both immobilized in Nylon membrane, in glycine buffer 0.1 M (pH 8): (1) to the addition of butyrylcholine, at 2×10^{-4} M final concentration; (2) to the addition of physostigmine at 0.55 μM final concentration.

Results

In this paper the enzyme system, sensor assembly and working conditions are substantially the same (Table 2) as those reported in previous papers [1, 2] in which the optimization of the response of two enzyme systems as a function of temperature, pH and substrate (butyrylcholine) concentration is described. Moreover, the choice of pH 8, as working pH, is particularly suitable in this case, as it is not only in agreement with the previous results of biosensor optimization [1], but also because it is a known fact [5] that, at this pH value the inhibiting action of the physostigmine is very strong. Nevertheless, we performed a further experimental optimization of the substrate concentration, due to the change of inhibitor compounds. For this purpose, in Fig. 2, the behaviour of the biosensor versus the substrate (butyrylcholine) concentration is shown with the physostigmine concentration in solution being maintained constant. Also a full electrochemical characterization of the biosensor and the behaviour of the biosensor response in the linearity concentration ranges, respectively in choline and butyrylcholine solutions, has been reported in a previous paper [2].

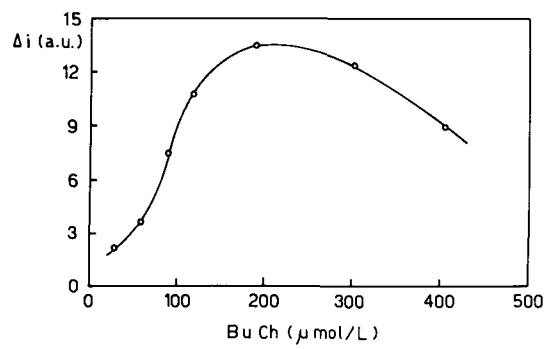

Figure 2
Optimization of the butyrylcholine inhibition enzyme sensor response, to physostigmine (0.63 μM), at several concentrations of substrate (butyrylcholine).

All the analytical data (response time, lifetime, linearity range, accuracy and precision) for the standard solutions of physostigmine analysis by the biosensor, are reported in Table 2 using butyrylcholinesterase or acetylcholinesterase enzyme, alternately. The response of the biosensor to physostigmine, over the whole concentration range is shown in Fig. 3; in addition, Fig. 4 explains the behaviour of the biosensor response during its lifetime. In Table 3 the reproducibility data of several calibration

Table 2
Main working conditions and analytical data of physostigmine analysis by the enzyme sensor (choline oxidase and butyrylcholinesterase, or acetylcholinesterase, both immobilized in Nylon membrane and butyrylcholine, or acetylcholine, 2×10^{-4} M as substrate)

	Using butyrylcholinesterase	Using acetylcholinesterase
Substrate	Butyrylcholine, 2×10^{-4} M	Acetylcholine 2×10^{-4} M
Temperature analysis	25°C	25°C
Buffer	Glycine 0.1 M, pH 8	Glycine 0.1 M, pH 8
Response time	3 min	5 min
Time of analysis	7 min	10 min
Lifetime	14 days; (able to work at least during 12 days)	14 days; (able to work at least during 10 days)
Regression line	$y = 12.30x + 4.67$ (y in a.u.; x in μM)	$y = 6.44x + 1.50$ (y in a.u.; x in μM)
Correlation coefficient	0.9999	0.9980
Linearity range	0.05–1.50 μM	0.4–1.2 μM
Precision on standard solutions (as RSD%)	<3.3%	<4.9%
Inaccuracy on standard solutions (by direct method)	−3.9–−3.7 (% values)	−1.4–+4.0 (% values)
Minimum detection limit	0.02 μM	0.2 μM

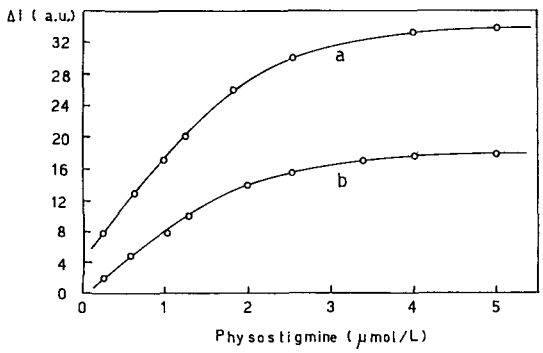

Figure 3
Response of the inhibition enzyme sensor to physostigmine in the whole concentration range. Enzymes both immobilized in Nylon membrane and using a hydrogen peroxide electrode as indicating sensor. Curve (a), using 2.0×10^{-4} M butyrylcholine as substrate; Curve (b), using 1.2×10^{-4} M butyrylcholine as substrate.

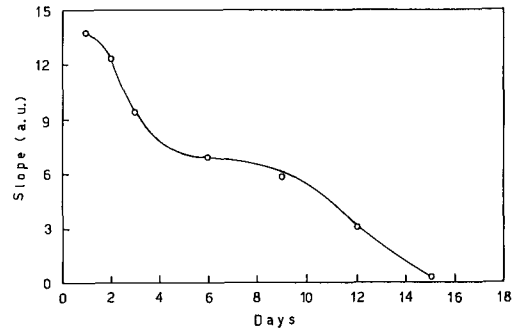

Figure 4
Lifetime of the inhibition enzyme sensor for compounds with anticholinesterase activity measurement, as slope value of the calibration graph vs days.

graphs for physostigmine analysis are summarized and, in Table 4, a comparison of calibration graph, response time and lifetime data are shown for several compounds with anticholinesterase activity.

In Table 5 and 6 the results of analysis in authentic pharmaceutical matrices are explained in detail. In Table 5 a comparison of the content, found by biosensor, of several substances showing anticholinesterase activity, contained in six different pharmaceutical preparations and the nominal content values of some drugs supplied by manufacturing firms, are shown; in addition, the precision data of measurements in these authentic matrices are reported in detail in the same table. In Table 6, the results concerning recovery tests, using the standard addition method, carried out on commercial pharmaceutical forms containing neostigmine, pyridostigmine, physostigmine and edrophonium, respectively, are summarized.

Table 3
Slope and correlation coefficient reproducibility of four calibration graphs, 7 days after preparation, using the butyrylcholine enzyme sensor, in standard solutions of physostigmine (butyrylcholinesterase and choline oxidase immobilized on Nylon membrane, butyrylcholine 2×10^{-4} M as substrate)

Calibration, *n*	Linearity range (μM)	Slope (a.u./μM)	Intercept (a.u.)	Correlation coefficient, *r*
1	0.06–1.3	6.57	3.43	0.9993
2	0.06–1.3	6.35	3.66	0.9991
3	0.06–1.3	6.22	3.52	0.9989
4	0.06–1.3	6.01	3.63	0.9995
Mean	0.06–1.3	6.28	3.56	0.9992
		(RSD% = 3.2)	(RSD% = 2.6)	

Table 4
Response time, lifetime and calibration graph for different drugs, with anticholinesterase activity, obtained using the enzyme sensor on the same day as preparation (choline oxidase and butyrylcholinesterase both immobilized in Nylon membrane and butyrylcholine 2×10^{-4} M as substrate)

Drug	Response time (min)	Linearity range (μM)	Slope (a.u./μM)	*y*-axis intercept (a.u.)	Correlation coefficient, *r*	Lifetime (days)
Physostigmine	<3.0	0.05–1.5	12.30	4.67	0.9999	14
Neostigmine	<3.5	0.05–1.5	13.99	4.33	0.9998	15
Pyridostigmine	<3.5	0.05–0.8	11.26	3.53	0.9987	12
Edrophonium	<3.5	0.05–1.5	13.75	5.41	0.9991	15

Minimum detection limit for all drugs is ≈0.2 μM. Re-equilibration time for the sensor is ≤3 min.

Table 5
Repeatability of different compounds with anticholinesterase activity determination, in commercial drugs, using the enzyme sensor and comparison of found results by nominal values

Drug No. and their pharmaceutical form	Nominal value (as % by wt), *a*	Value found by enzyme sensor (mean of four determinations), *b*	RSD (%)	$(\frac{b-a}{a})\%$
1 (Ampoules)*	0.050	0.051	0.2	+2.0
2 (Tablets)*	6.52	6.51	1.0	−0.15
3 (Ampoules)†	0.100	0.103	0.4	+3.0
4 (Tablets)†	17.1	17.08	4.3	−0.12
5 (Syrup)‡	0.100	0.104	0.4	+4.0
6 (Ampoules)§	1.00	1.03	3.4	+3.0

* Containing neostigmine.
† Containing pyridostigmine.
‡ Containing physostigmine.
§ Containing edrophonium.

Discussion

The concentration of the substrate has a decisive effect on the level of inhibition that the inhibitor can exert on the butyrylcholinesterase enzyme [2] because the inhibition is competitive [5]. In Fig. 2, the sensor response, at a constant inhibitor concentration, is shown to be a function of the concentration of the substrate. Furthermore, the higher inhibition and consequently the stronger response of the inhibition sensor, are obtained with a substrate concentration of about 2×10^{-4} M. It is interesting to note that this concentration of butyrylcholine is practically the same as that found previously [2] when sensor response was optimized for organophosphorus pesticides. Despite this, there are significant differences compared with the results of pesticide determination: in particular, sensor response time (and thus also analysis time) is much shorter in this case than in the previous one [2] (3 min instead of 10). This implies that the inhibition process is faster and that also the time required to restore normal sensor response after an inhibition measurement is shorter (about 3 min instead of 10) [2]. Regeneration, which is carried out in 2×10^{-4} M butyrylcholine solution, is thus more rapid and complete than in the case of pesticide analysis. This is to be expected since, in this case, as is known [5], inhibition is much more reversible than in the case of organophosphorus pesticides. Moreover, as pointed out previously [2, 6], the use of immobilized butyrylcholinesterase enzyme rather than a homogeneous solution of this, seems in any case to increase the degree of reversibility of the inhibition process.

The behaviour of the sensor response, towards increasing physostigmine concentrations and over a wide range of concentrations, is shown in Fig. 3. It is seen to be typical of an enzyme inhibition sensor [2, 7, 8]. Furthermore, the two curves in Fig. 3 provide additional evidence of the important rôle played by the concentration of the substrate used, on the extent of inhibition sensor response. As can be seen, from Table 4, the linearity range is about one decade and half, i.e. about half a decade wider than that found in organophosphorus pesticide analysis [2], while the minimum detectable limit is practically the same in both cases (about 0.02 μM). Likewise, also the accuracy and precision values are of the same order, while the correlation coefficient value is better in the physostigmine determinations (Table 2) than in pesticide dosage [2]. Table 2 also shows a comparison of the main analytical data referring to physostigmine determination according to whether the butyrylcholinesterase or acetylcholinesterase enzyme is used (of course by employing the proper substrate too, i.e. butyryl-, or acetylcholine, respectively). It can be seen how the former enzyme is to be preferred to the latter, both as far as response time and lifetime, as well as sensitivity, useful range and minimum detectable limit are concerned. This confirms, also for this case, the observations made in previous papers [1, 9]. Examination of Table 3 also shows that the reproducibility of the calibration curves in standard physostigmine solutions is certainly satisfactory. On the other hand, as shown by analysis of the data summed up in Table 4, the main analytical characteristics found in physostigmine

Table 6
Recovery of different compounds, with anticholinesterase activity, in commercial drugs, using the enzyme sensor. Samples were diluted appropriately before each measurement (values in μM)

Drug No. and their pharmaceutical form	Value found in the drugs (mean of four determinations)	Added		Total value (found + added), *a*	Experimental total value (mean of three determinations), *b*	$\left(\frac{b-a}{a}\right)\%$
1 (Ampoules) containing neostigmine	0.75	Neostigmine	0.40	1.15	1.20	+4.3
2 (Tablets) containing neostigmine	0.77	Neostigmine	0.40	1.17	1.21	+3.4
3 (Ampoules) containing pyridostigmine	0.44	Pyridostigmine	0.20	0.64	0.61	−4.7
4 (Tablets) containing pyridostigmine	0.46	Pyridostigmine	0.20	0.66	0.68	+3.0
5 (Syrup) containing physostigmine	0.81	Physostigmine	0.40	1.21	1.24	+2.5
6 (Ampoules) containing edrophonium	0.69	Edrophonium	0.40	1.09	1.06	−2.8

measurement, using the inhibition sensor and set out in Table 2, as well as the plot of sensor response vs lifetime (Fig. 4), closely resemble those obtained in the determination, by the inhibition sensor, of other drugs with anticholinesterase properties, such as neostigmine, pyridostigmine and edrophonium. Naturally no selectivity among these pharmaceutical substances with anticholinesterase activity is observed. On the other hand, this fact does not reduce the possibility to apply this sensor in the analysis of pharmaceutical forms such as those listed in Table 1, because only one anticholinesterase drug is contained in each one of these commercial forms. Moreover the very close similarity of the sensor response to different anticholinesterase drugs makes it possible to measure, if necessary, the total level of anticholinesterase activity of a mixture containing several anticholinesterase drugs.

Detailed data have already been published in a previous paper [2] concerning sensor response to other substances showing anticholinesterase activity, but having importance in the environmental field, e.g. several organophosphorus pesticides and carbamates [8, 10], rather than in the pharmaceutical field. Other possible interferents in the analysis by new inhibition enzyme sensors are listed in the same paper [2]. We observed that fluorides, Pb^{2+} and nicotine, which have a notable effect on sensor response, should not be considered true interferents, except at very high concentrations.

If used for routine analysis of butyrylcholine, the sensor has a lifetime of at least 2 weeks (no more than 20% of the original activity is lost during the first 12 days). From Fig. 4 it can be observed that, if the sensor is used to determine inhibitors, between the third and ninth day after preparation, the response is about constant, as also shown by the good reproducibility of the calibration graphs (Table 3) recorded at the seventh day. However the correct way of using the biosensor is to compare its response to the sample with that to a standard of similar concentration, taking care that both values fall within the linearity range. In such a way reliable determinations can be performed, even if a certain decrease of the response is observed, due to the loss of enzyme activity [4].

Conclusions

The results contained in Tables 5 and 6, in which data obtained from the analysis of six different commercial pharmaceutical forms are explained in detail, with regard both to the repeatability and accuracy of measurements and the correlation with nominal values supplied by producer firms, show that the analysis of pharmaceutical preparations, using the enzyme inhibition sensor, is possible and gives satisfactory reproducibility and accuracy without remarkable pretreatments of the samples. The solubilized drugs were added directly (or after a proper dilution), under stirring, to the buffer glycine solution containing butyrylcholine thermostatted in a glass cell and the signal variation of the amperometric biosensor, dipping into the buffer, recorded. The absence of other significant interfering agents, since the number of substances with anticholinesterase activity is very small, and the possibility of directly analysing, without pretreatment, even turbid or coloured solutions, contribute to make this analytical method one of theoretical and practical interest. It is also true that the comparison with other electroenzyme methods previously developed by us [1, 9] is favourable to the method using the inhibition sensor described herein.

Nevertheless, it is important to observe that the indicating system (in practice the choline enzyme sensor) used in the assembly of the inhibition sensor is characterized by high specific response and works in well buffered solutions. It is thus unaffected by pH change resulting, for example, from the authentic sample to be analysed. This is a great advantage of this sensor with respect to other electrochemical systems using less specific indicating electrodes, implemented by other authors in recent years [11–14].

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References

[1] L. Campanella, M. Achilli, M.P. Sammartino and M. Tomassetti, *Bioelectrochem. Bioenerg.* **26**, 237–249 (1991).
[2] L. Campanella, R. Cocco, M.P. Sammartino and M. Tomassetti, *Sci. Tot. Environ.* **123/124**, 1–16 (1992).
[3] M. Mascini and M. Mazzei, *Anal. Chim. Acta* **192**, 9–16 (1987).
[4] M. Mascini, M. Tomassetti and M. Iannello, *Clin. Chim. Acta* **132**, 7–15 (1983).

[5] W.C. Bowman and M.J. Rand, *Farmacologia* (It. Ed.). Medical Science International, Roma (1985).
[6] M. Bernabei, C. Cremisini, G. Palleschi and M. Mascini, IX Congr. Naz. Div. Chim. Anal. S.C.I., Ferrara, 28 October 1990, Atti, p. 236 (1990).
[7] F. Schubert, R. Renneberg, F.W. Scheller and L. Kirstain, *Anal. Chem.* **56**, 1677–1682 (1984).
[8] U. Wollemberger, K. Setz, F.W. Sheller, U. Loffler, W. Gopel and R. Gruss, *Sens. Actuators* **B4**, 257–260 (1991).
[9] M. Achilli, L. Campanella, M.P. Sammartino and M. Tomassetti, *Inquinamento*, **November** (**11**), 66–70 (1990).
[10] J.L. Marty, R. Rouillon, K. Sode and I. Karube, *'Biosensor 90', Singapore 2 May 1990*, Abstr. p. 85. Elsevier, Amsterdam (1990).
[11] G.G. Guilbault and J. Kristoff, *Anal. Chem.* **57**, 1754–1756 (1985).
[12] H. El Yamani, C. Tran-Minh, A. Abdul and D. Chavanne, *Sens. Actuators* **15**, 193–198 (1988).
[13] C. Tran-Minh, P.C. Pandey and S. Kumaron, *Biosens. Bioelectr.* **5**, 461–471 (1990).
[14] R. Gruss, F. Scheller, M.J. Shao and C.C. Liu, *Anal. Lett.* **22**, 1159–1169 (1989).

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