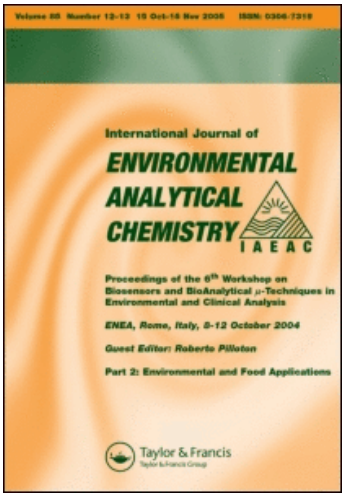

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Advantages and limitations of a novel hybrid biosensor for detecting toxic compounds in food

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Advantages and limitations of a novel hybrid biosensor for detecting toxic compounds in food

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A hybrid biosensor for rapid detection of several toxic compounds in foods is hereby described. This device employs a non-invasive electrochemical sensor based on a potentiometric gas diffusion carbon dioxide electrode coupled with specific microbial cultures. Determinations are based on the perturbation of the respiration activity of an appropriate microorganism in the presence of different toxic compounds. *Escherichia coli* and *Bacillus stearothermophilus* strains that were susceptible to multiple antibacterial drug residues, and the *Saccharomyces cerevisiae* yeast that was susceptible to multiple toxic compounds, were employed as sensitive cells. The analyte considered for indirectly measuring antimicrobial inhibition by toxic agents was carbon dioxide (CO_2). The electrical signal detected is related to the amount of CO_2 developed during bacterial respiration. The methodological approach compared to older screening methods, also inhibition based, offers the advantages of (i) low cost; (ii) shorter analysis time; (iii) smaller sample amount; (iv) no sample treatment; (v) good precision; and (vi) the possibility of following, in a continuous manner, the inhibition process.

Keywords: Electrochemical sensor; Antimicrobial drug residues; Heavy metals; Food analysis

1. Introduction

The occurrence of violative residues of veterinary medicine and other toxic compounds in food of animal origin is an issue of primary interest within the European Union [1, 2].

For an accurate risk assessment involved in contaminated food consumption it is necessary to have suitable methods for monitoring programs. The achievement of low-cost, simple and reliable analytical methods for analysis of contaminants and residues in food, at trace and ultratrace levels, allows us to obtain information and knowledge which is able to solve the specific problems regarding human health [3, 4].

Current methods of detecting these compounds are based on separation techniques (i.e., HPLC, GC, CZE, etc.) with different detection techniques (i.e., UV-Vis, DAD, fluorescence, MS, MS/MS, etc.) that are sometimes expensive, time consuming and

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not useful for field analyses. In this regard, it is opportune to underline the fact that the recent introduction of hazard analysis critical control points, more commonly known as HACCP, in food production, has further increased the demand for analytical methods that are fast and able to operate on-line.

Biosensors could play an important role in this regard and a growing interest for electrochemical biosensor techniques for detecting contaminants and residues in food has been observed in recent years [5–8].

A biosensor comprises two distinct elements: a biological recognition element (e.g., antibodies, enzymes, lectins, receptors, microbial cells, etc.) and in close contact, a signal transduction element (e.g., optical, acoustic or electrochemical) connected to a data acquisition and processing system [9].

This article describes a novel application for a hybrid biosensor developed for detection of some contaminants and residues that are of concern to human health. In particular, antimicrobial drug residues and several heavy metals (i.e., cadmium, mercury and lead), have been considered as being of particular concern for consumer health.

The new method employs a non-invasive electrochemical sensor based on a potentiometric gas diffusion carbon dioxide electrode coupled with sensitive microrganisms. The effect of toxic compounds on respiratory activity of microrganisms can be employed to detect their presence in foodstuffs [10, 11].

In this study, the analyte investigated is carbon dioxide, the production rate of which has a direct relationship with the inhibition of sensitive cell growth [12]. Each microorganism is, in fact, supposed to produce CO_2 by respiration at the constant rate (G, mol s^{-1}); therefore, the overall CO_2 production rate (v_{CO_2}, mol s^{-1}) is given by:

$$v_{CO_2} = Gn$$

where n is the number of microrganism.

The CO_2 produced by living microrganisms is stripped, by means of a defined gaseous flow at known concentration rate, from the culture medium (contained in the analysis cells) and carried to the sensor, which develops an emf whose value (E, mV) depends on the CO_2 gaseous concentration (y_{CO_2}, μL L^{-1}) by:

$$E = E^\circ + K \log(y_{CO_2})$$

where E° and K are experimental values obtained from the calibration procedure with gaseous standards (i.e., air samples having known y_{CO_2} values).

The following relations hold [4]:

$$y_{CO_2} = v_{CO_2} \frac{RT}{F_g} = \frac{GnRT}{F_g}$$

$$E = E^\circ + K \log\left(\frac{GRT}{F_g}\right) + K \log(n)$$

where F_g is gaseous flow rate (L s^{-1}), T is the absolute temperature (K) and R is the gas constant.

2. Experimental

2.1 *Equipment*

The Hybrid BioSensor (HyBS) employed in this study is derived from an already described prototype [13]. The electrochemical component was a galvanic cell composed of a reference electrode dipped in electrolytic solution and connected to a stainless steel indicator electrode by means of a silk filament that acts as a sensor membrane (figure 1). It is contained in a case-box, protecting it from electrical straw currents and drafts. The case-box is connected by steel tubes with the carrier gas (CG) source (air compressed in the cylinder), and the analytical glass cells specifically realized (figure 2). The HyBS is connected to an emf-meter connected in series with a computer for data acquisition and elaboration (figure 3).

The functioning of HyBS is based on:

1. measurement of the emf (E, mV) of a tensiometric galvanic cell composed of an indicator electrode (IE), a reference electrode (RE) and a working electrolytic solution (WS);
2. dependence of E on the WS composition;
3. dependence of WS composition on the y_{CO_2} value of the gaseous flow impacting the sensor filament (SF);
4. CO_2 permeation through SF, its dissolution in WS, modification of its content;
5. IE is a metallic corrosion electrode, the most used electrode material being stainless steel. RE is either a conventional electrode or a corrosion electrode like IE. WS is an immobilized solution; its composition fits the y_{CO_2} level, and avoids interferences from other gaseous chemical species.

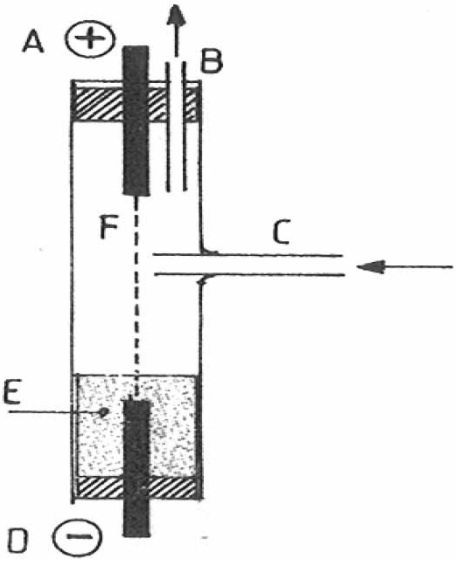

Figure 1. CO_2 sensor design: (A) indicator electrode, (B) carrier gas outlet, (C) carrier gas inlet, (D) reference electrode, (E) electrochemical solution, (F) sampling probe (silk filament).

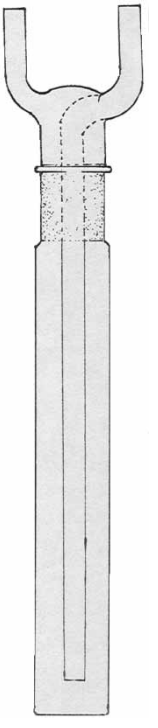

Figure 2. Analytical cell: (A) carrier gas outlet, (B) carrier gas inlet.

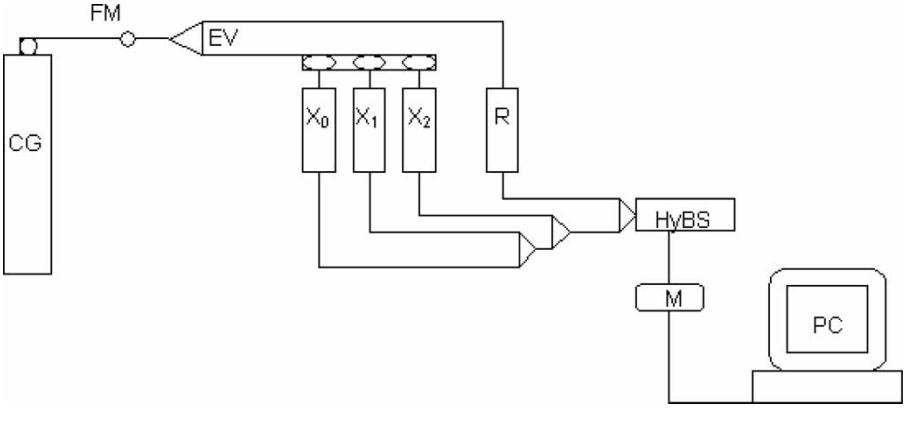

Figure 3. Block and flow diagram of HyBS: (CG) carrier gas source (atmospheric air in cylinder), (EV) electromagnetic valves, (HyBS) hybrid biosensor, (X) and (R) analysis and reference cells, (FM) flow meter, (M) multimeter.

2.2 *Gas reference materials (GRMs)*

Four GRMs of CO_2 (SIAD Srl, Rome, Italy) at concentrations of 1253, 1745, 2881, 6600 ppm were employed for sensor calibration [8].

2.3 *Microorganisms*

Experiments have been carried out employing as sensitive microrganism, cells of *Escherichia coli* and *Bacillus stearothermophilus* that were susceptible to multiple antibiotics, and *Saccharomyces cerevisiae* yeast that is susceptible to multiple toxic compounds, including heavy metals [14–17]. The microorganism used throughout this work was *E. coli* ATCC 11303 (ATCC, Rockville, MD, USA). The cells were stored at 4°C on Mueller-Hinton agar slants and grown at 37°C in Mueller-Hinton broth (Oxoid Ltd, Basingstoke, Hampshire, England). The other microrganism used was *B. stearothermophilus* var. *calidolactis* ATCC 10149 (ATCC, Rockville, MD, USA). The cells were stored at 4°C on Mueller-Hinton agar slants and grown at 65°C in Mueller-Hinton broth (Oxoid Ltd, Basingstoke, Hampshire, England). The yeast employed was *S. cerevisiae* ATCC 9763 (ATCC, Rockville, MD, USA). The cells were stored at 4°C and grown at 30°C in YEPD (Oxoid Ltd, Basingstoke, Hampshire, England).

All the cultures were standardized at $5 \times 10^4\,mL^{-1}$ by means of UV spectrometry measurements (Perkin Elmer Instruments LLC, Shelton, USA).

2.4 *Standards*

Standards of twenty-six antibacterial drugs were all supplied by Sigma Chemical (Sigma-Aldrich, Milan, Italy). Standard stock solutions in water ($10\,mg\,L^{-1}$) of each drug were prepared and stored at −18°C. Spiking solutions of all drugs were prepared by diluting suitable volumes of each standard stock solution with water. Standards of Cd, Hg and Pb were supplied by Merck (VWR International, Milan, Italy). Spiking solutions of all metals were prepared by diluting suitable volumes of each standard stock solution with bi-distilled water.

2.5 *Sample preparation*

The samples were prepared by adding to 4 mL of cultural medium, 0.5 mL of bacterial culture standardized ($1 \times 10^7\,mL^{-1}$) as inoculum and 0.5 mL of distilled water, in the case of control samples, or 0.5 mL of spiking solution at different concentrations for spiked samples.

2.6 *Analysis*

The twenty-six antibacterial drugs and the three heavy metals have been tested at concentrations ranging from half to ten times the lowest maximum level fixed for these toxic compound in foods.

Instrumentation employed and adopted working conditions are shown in table 1. The carrier gas (CG) was left to flow at a constant rate of $0.1\,mL\,s^{-1}$ through the reference cell, containing 5 mL of cultural medium, and the HyBS in series, until a constant value of the baseline ($E°$, mV) was achieved; then the CG was diverted in succession through the sample cells containing the same cultural medium and the toxic compound under investigation which then reached the sensor. Potential difference (ΔE) *versus* time values were registered for 180 min in steps of 30 min. Analytical cycles

Table 1. Instrumentation and working conditions for Hybrid-BioSensor (HyBS).

	Instrumentation
Galvanic cell	Reference electrode dipped in electrolytic solution and connected to a stainless steel indicator electrode by means of a silk filament that acts as a sensor membrane
Linearity range:	From 10 μL L^{-1} to 10^4 μL L^{-1}
k value:	About 20 mV
Resolution:	0.5 μL L^{-1} at 400 μL L^{-1} Level
Signal noise:	1–2 μV
Thermal drift:	About 20 μV $°C^{-1}$
The lifetime:	About 1500 detections
Analytical cell	Glass vial height 15 cm; inside diameter 2 cm
Potentiometer	Multimeter Hewlett-Packard 34401A
Thermostatic water bath	GFL 1002 Digit
Carrier gas source	Compressed air in cylinder
Software	Agilent Intui Link, Hewlett-Packard
	Working conditions
Biological recognition element and grown conditions	*Escherichia coli* ATCC 11303 Müeller Hinton broth (Oxoid, Basingstoke, UK); 37°C ± 1 *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 Müeller-Hinton broth (Oxoid, Basingstoke, UK) 63°C ± 1 *Saccharomyces cerevisiae* ATCC 9763 Yeast extract, 1%, peptone, 2%, glucose 2%; 30°C ± 1
Standards	28 antibiotic: tetracyclines, quinolones, penicillins, aminoglycosides, macrolides, sulphamides; lead (Pb), mercury (Hg), cadmium (Cd)
Carrier gas	Air
Flow	0.1 mL s^{-1}
Incubation time	
Antibiotics	180 min
Heavy metals	120 min
Reading-time	1 min
Gas reference materials (GRMs)	Four GRMs of CO_2 (SIAD Srl, Rome, Italy) at concentrations of 1253, 1745, 2881, 6600 μL L^{-1}

for a total duration of 5 min were realized as follows: 1 min of analysis and 4 min for the sensor re-equilibration (the CG flow through the reference cell).

2.7 *In-house method validation*

Method trueness was evaluated on the basis of recoveries by means of the four gas reference materials (GRMs) at different CO_2 concentrations. Precision, expressed as repeatability, was calculated by repeated analyses ($n = 8$) on the same sample sets used for recovery tests.

2.8 *Statistical analysis*

The results obtained from each tested substance were subjected to both parametrical and non-parametrical statistical analysis. Furthermore, in order to evaluate the significance of the differences noted among the different concentrations of toxic *versus* blank,

the multi-factor analysis of variance (ANOVA test) was applied. Statgraphics software (ver. 7 for DOS, Manugistic) was used for processing.

3. Results and discussion

3.1 *Performance of the analytical method*

Results of the in-house method validation are shown in table 2. Analytical determinations of GRMs showed good precision with a relative coefficient of variation (CV%) between 0.8 and 2.4%.

These data indicate that the repeatability of the method used in this study is good and the recovery data were also satisfactory with values ranging from 97.4 to 105.8%. The value of analytical signal (mV) has shown to be directly proportional to CO_2 concentration in the range between 1253 and 6600 $\mu L\,L^{-1}$. The relative plot with correlation equation and squared correlation coefficient are reported in figure 4.

In general, it was possible to detect all antibacterial molecules taken into consideration and the three heavy metals. For detection of quinolones and tetracyclines, *E. coli* was the most suitable sensitive element, whereas for the other antibiotic classes (penicillins, macrolides, sulphonamides, aminoglycosides) resulted in *B. stearothermophilus* being the most suitable. As regards the heavy metals *S. cerevisiae* yeast was the more suitable sensitive element.

Table 2. Performance of the analytical method.

	CO_2 content ($\mu L\,L^{-1}$)		Trueness	Precision
Gas reference material	Certified	Found (mean ± SD)	Recovery (%)	CV (%)
MRG 1	1253	1220.36 ± 0.06	97.39	2.0
MRG 2	1745	1829.72 ± 0.06	104.8	1.7
MRG 3	2881	3047.90 ± 0.04	105.8	0.8
MRG 4	6600	6766.09 ± 0.22	102.5	2.4

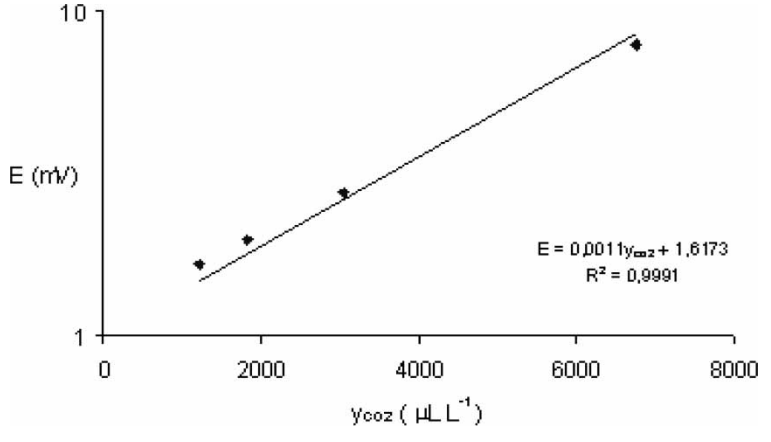

Figure 4. Correlation equation and squared correlation coefficient.

The results of antibacterial drugs analysis has shown that *E. coli* was sensitive to quinolones and tetracyclines whereas the other antibacterials did not show statistical significant bacterial inhibition even at concentrations ten times higher than the lowest MRL. On the other hand, *B. stearothermophilus* is shown to be sensitive to penicillins, macrolides, sulphonamides, aminoglycosides and tetracyclines but not quinolones. Tetracyclines were effective for both employed strains. Thus, this behaviour allows for the presumptive identification of three groups of antibacterial molecules: quinolones, tetracyclines and all the others.

Meaningful differences of the degree of inhibition were already in evidence after 120 min. The degree of inhibition continued to increase up to 180 min. After this time there were no significant variations of ΔE. In terms of percent ΔE decrease,

Table 3. ΔE and inhibition percentage (values are mV, mean ± SD for $n = 6$ independent samples).

	Measurements to 120 min			
Antibiotic	Lowest MRL ($\mu g\,kg^{-1}$)	Control ΔE (mV)	Sample ΔE (mV)	Inhibition (%)
Tetracyclines[a]				
Tetracycline	100	3.67 ± 0.16	1.89 ± 0.11	−49**
Oxytetracyline	100	3.47 ± 0.20	1.82 ± 0.27	−48**
Chlorotetracycline	100	3.90 ± 0.19	1.82 ± 0.32	−53***
Quinolones[a]				
Norfloxacin	100	3.65 ± 0.22	1.93 ± 0.19	−49**
Ciprofloxacin	100	3.64 ± 0.17	1.16 ± 0.24	−68***
Enrofloxacin	100	4.10 ± 0.21	1.69 ± 0.22	−59**
Flumequine	50	3.79 ± 0.20	0.86 ± 0.12	−78***
Nalidixic acid	–	4.49 ± 0.29	2.15 ± 0.21	−52**
Marbofloxacin	75	3.83 ± 0.31	0.89 ± 0.14	−77***
Danofloxacin	30	4.00 ± 0.21	1.44 ± 0.18	−64***
Penicilline[b]				
Ampicillin	4	2.99 ± 0.24	1.05 ± 0.22	−65***
Penicillin G	4	3.34 ± 0.23	1.17 ± 0.24	−67***
Amoxicillin	4	3.14 ± 0.18	0.97 ± 0.29	−69***
Cloxacillin	30	3.30 ± 0.15	1.15 ± 0.26	−69***
Aminoglycosides[b]				
Streptomycin	200	3.27 ± 0.27	1.80 ± 0.19	−51**
Neomycin	500	3.05 ± 0.30	1.60 ± 0.23	−52**
Gentamicin	50	3.10 ± 0.28	1.68 ± 0.22	−54**
Spectinomycin	200	3.40 ± 0.34	1.89 ± 0.30	−49**
Macrolides[b]				
Erythromycin	40	3.10 ± 0.32	1.30 ± 0.25	−55**
Tylosin	50	2.29 ± 0.16	1.22 ± 0.28	−59***
Spiramycin	200	2.98 ± 0.28	1.28 ± 0.31	−57**
Tilmicosin	50	3.12 ± 0.27	1.40 ± 0.21	−55**
Sulphonamides[b]				
Sulfadiazine		2.88 ± 0.26	1.06 ± 0.18	−63***
Sulfadimethoxine	100	3.05 ± 0.24	1.01 ± 0.19	−67***
Sulfathiazole	100	3.12 ± 0.21	1.06 ± 0.22	−66***
Sulfamethazine	100	3.45 ± 0.22	1.03 ± 0.28	−70***
Heavy metals[c]				
Cadmium	1	2.78 ± 0.28	0.63 ± 0.32	−23*
Mercury	1	2.70 ± 0.25	0.11 ± 0.28	−41**
Lead	1.5	2.75 ± 0.32	0.99 ± 0.27	−36*

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as found by ANOVA (spiked samples *vs.* correlated control).
Microrganisms employed: [a] *E. coli*; [b] *B. stearothermophilus*; [c] *S. cerevisiae*.

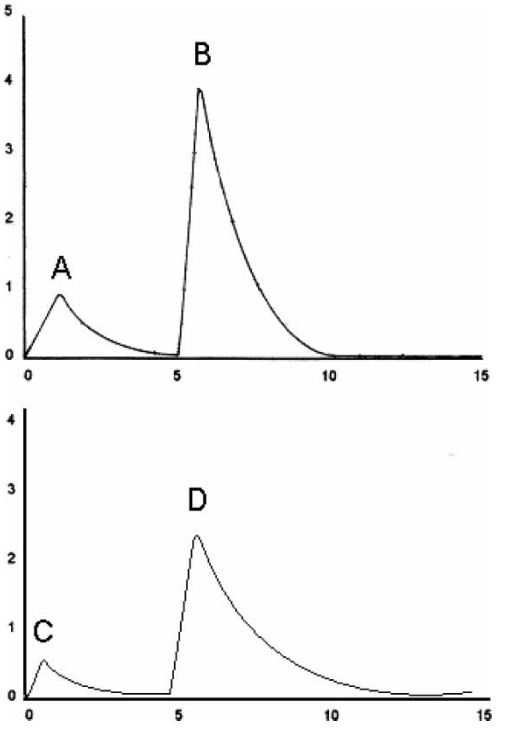

Figure 5. Example of two recorded *E vs.* time curves: (A) flumequine ($100\,\mu g\,kg^{-1}$), (B) control (*E. coli*); (C) cadmium ($1\,\mu g\,kg^{-1}$), (D) control (*S. cerevisiae*).

the antibiotic that, after 120 min, has shown the highest inhibitory effect at its lowest MRL ($25\,\mu g\,L^{-1}$) has been the flumequine (−78%) whereas the lowest inhibition has been displayed by oxytetracycline (−48%).

Results of all specimen sensorial analysis are shown in table 3 while figure 5 shows two examples of the *E versus* time recorded curve.

4. Conclusions

The methodological approach compared to older screening methods, also inhibition based, offers the advantages of (i) low cost; (ii) shorter analysis time; (iii) smaller sample amount; (iv) no sample treatment; (v) good precision; (vi) large linearity range; and (vi) the possibility of following, in a continuous manner, the inhibition process.

The low detection limit suggests that the method could be used for drug residue and toxic compound determination in food, although the sensitivity to a wide range of substances makes it much less specific. For this reason we are evaluating the possibility of using different combinations of working parameters (strains, medium, pH, activator and inhibitor substances) to improve the specificity.

In any case, given the encouraging results obtained in this pilot study, other main classes of toxic compounds will be investigated in the future. Different food matrices will be taken into consideration. This could constitute a notable starting point for

sound and effective action in replacing older screening detection methods, normally employed for toxic residue detection, with a simple, fast and sensitive method so as to enhance food control and monitoring in the toxic residue field.

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