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Conductometric tyrosinase biosensor for the detection of diuron, atrazine and its main metabolites

Tuan Mai Anh [a,b], Sergei V. Dzyadevych [c], Minh Chau Van [d], Nicole Jaffrezic Renault [e], Chien Nguyen Duc [b], Jean-Marc Chovelon [a,*]

[a] *Laboratoire d'Application de la Chimie à l'Environnement, UMR CNRS 5634, Université Claude Bernard Lyon I, 43 Boulevard du 11 Novemvre 1918, Villeurbanne Cedex 69622, France*
[b] *International Training Institute for Materials Science (ITIMS), Hanoi University of Technology, 1 Dai Co Viet, Hanoi, Viet Nam*
[c] *Laboratory of Biomolecular Electronics, Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, 150 Zabolotnogo Street, Kiev 03143, Ukraine*
[d] *Institute of Natural Products Chemistry, Vietnam National Centre for Science and Technology, Hoang Quoc Viet Street, Hanoi, Viet Nam*
[e] *IFoS FRE 2608, Ecole Centrale de Lyon, 36 av. Guy de Collongue, 69134 Ecully Cedex, France*

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Abstract

The determination of diuron, atrazine, desisopropylatrazine (DIA) and desethylatrazine (DEA) were investigated using conductometric tyrosinase biosensor. Tyrosinase was immobilised on the biosensor sensitive part by allowing it to mix with bovine serum albumin (BSA) and then cross-linking in saturated glutaraldehyde (GA) vapour for 30 min. The determination of pollutants in a solution was performed by comparison of the output signal (i.e percentage of the enzymatic activity) of the biosensor before and after contact with pollutants. The measurement of the enzymatic activity was performed using 4-chlorophenol, phenol and catechol substrates and response times ranging from 1 to 5 min were observed. A 4-chlorophenol substrate was used to detect pesticides. A 30 min contact time of the biosensor in the pollutant solution was used. Under the experimental conditions employed, detection limits for diuron and atrazine were about 1 ppb and dynamic range of 2.3–2330 and 2.15–2150 ppb were obtained for diuron and atrazine, respectively. A relative standard deviation ($n = 3$) of the output signal was estimated to be 5% and a slight drift of 1.5 $\mu S\,h^{-1}$ was observed. The 90% of the enzyme activity was still maintained after 23 days of storage in a buffer solution at 4 °C.

Keywords: Conductometric biosensor; Tyrosinase; Atrazine; Diuron

1. Introduction

Intensive industrialisation and the use of chemicals in agriculture—which are themselves consequences of successful economic and political measures to increase prosperity—have contributed to the presence of many toxic compounds in air, soil and water, causing environmental degradation and sometimes direct health hazards. The most common pollutants most often found during control analysis are atrazine, simazine, phenylureas herbicides, glyphosate, bromoxynil, paraquat among others and their degradation products.

Since the polarity and other chemical and physical properties of the compounds listed above vary considerably, several different analytical methods are used to detect the pollutants. A variety of extraction techniques have been employed over the years including liquid–liquid extraction, solid-phase extraction and solid-phase micro-extraction among others, followed by a quantitative analysis using various chromatographic techniques such as gas and liquid chromatography (GC and LC) [1–5]. To date, gas chromatography–mass spectroscopy (GC–MS) is the most commonly used analytical technique. All of the experimental methods are well established and exhibit a large spectrum of advantages. However, they are under constant improvement, time-consuming, expensive, cannot be performed easily outside the laboratory and need very highly skilled technicians. On the other hand, early-warning systems are needed for scientists to react in case of accidental pollution.

Enzyme-based biosensors for pollutant determination represent potential alternatives to the time-consuming and cum-

* Corresponding author. Tel.: +33-4-7243-2638; fax: +33-4-7244-8333.
E-mail address: chovelon@univ-lyon1.fr (J.-M. Chovelon).

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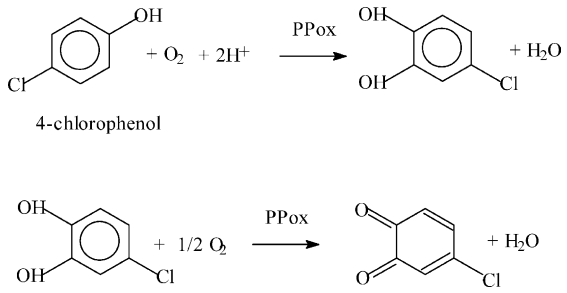

Fig. 1. Schematic reaction with tyrosinase.

bersome analysis of pollutants in the environment. In addition, enzyme-based biosensors act as early warning systems thanks to their unique characteristics: (1) selectivity, (2) relatively low cost of fabrication, (3) good storage stability and (4) potential for miniaturisation and easy automation, which could lead to the construction of simple portable devices for fast screening purposes and for in-field/on-site monitoring. In particular, several biosensors based on tyrosinase were developed for determination of phenols [6–15]. The tyrosinase was immobilised on an electrode's surface as a thin film or in a membrane on a Clark oxygen electrode [6,7], chemically bonded to a solid graphite electrode [8,9] or controlled-pore glass [10] and using electropolymerisation of an amphilic pyrrole derivative–enzyme mixture [11]. Tyrosinase is a binuclear copper containing metalloprotein which catalyses, in the presence of molecular oxygen, two different reactions: (1) the transformation of *o*-mono-phenols into catechols (Fig. 1, part 1, cresolase activity) and (2) the oxidation of catechol to *o*-quinone (Fig. 1, part 2, catecholase activity). In this context, these reactions can be monitored by various kinds of electrochemical detection techniques such as (1) detection of dioxygen consumption [12], (2) direct reduction of generated *o*-quinone [13] and (3) mediated reduction of *o*-quinone by hexacyanoferrate (II) [8].

In recent publications [14,15] a tyrosinase biosensor based on pH-sensitive field-effect transistors for phenol determination in water solution has been described. The main analytical characteristics were studied under different conditions, as well as the possibility of optimising these working parameters. Different factors, such as pH of immobilisation, the enzyme loading and time of immobilisation in glutaralaldehyde vapour were investigated with respect to the influence on sensitivity, limit of detection, dynamic range, operation and storage stability.

On the other hand, it has also been reported that triazine and phenylurea herbicides could be determined from the inhibition of tyrosinase activity to a substrate using amperometric biosensors [16–21]. For example Vedrine et al. [21] have found limit of detection for atrazine and diuron in range of 1–0.5 ppm, respectively using dopamine as substrate which agree with those given by other authors mentioned above. To our best knowledge, no previous work describing tyrosinase inhibition by these pollutants using conductometric sensors has been reported to date. Conductometric sensors for biosensing devices have been introduced by Watson et al. [22]. The device consisted of an oxidised silicon wafer with interdigitated gold electrode pairs on one surface in a planar configuration. The principle of the detection is based on the fact that many biochemical reactions in solution produce changes in the electrical resistance (reciprocal conductance). Conductance measurements involve the resistance determination of a sample solution between two parallel electrodes. This is a unique method for a direct assaying of many enzymes and their substrates. According to Lawrence [23] five factors allow the application of conductometric methods for enzymatic analysis: (1) generation of ionic groups (e.g. amidases); (2) separation of unlike charges (e.g. decarboxylases); (3) proton migration (e.g. esterases); (4) changes in the degree of association of ionic groups resulting from chelation (e.g. kinases); and (5) changes in the sizes of charge-carrying groups (e.g. phosphatases). In addition, a conductance change can be observed when mobility in the solution of the species is changed. Conductometric biosensors were tested in the detection of urea in human serum samples [22] and for the enzymes penicillinase and acyl amilamidohydrolase activity studies [24].

Conductometric biosensors present a number of advantages: (1) thin-film electrodes are suitable for miniaturisation and large scale production using inexpensive technology, (2) they do not require a reference electrode, (3) transducers are not light sensitive, (4) the driving voltage can be sufficiently low to decrease significantly the power consumption, (5) large spectrum of analytes of different nature can be determined on the basis of various reactions and mechanisms.

In this work a conductometric tyrosinase biosensor for the detection of diuron, atrazine and its main metabolites, desethylatrazine and desisopropylatrazine (DEA, DIA), is described. Experimental parameters such as the incubation time in pollutant solutions or the choice of the substrate have been determined. Promising results have been obtained since limits of detection were in the parts per billion range for both atrazine and diuron. Such limits of detection are better than the ones obtained using amperometric tyrosinase biosensor described in the literature.

2. Experimental

2.1. Reagents

Tyrosinase (polyphenol oxidase; PPox) (EC1.14.18.1, 6680 U/mg) from mushroom, bovine serum albumin (BSA), 4-chlorophenol, phenol, catechol and aqueous solutions (25% w/v) of glutaraldehyde (GA), atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) (CAS: 1912-24-9, EC 2176178), diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) (CAS: 330-54-1, EC: 2063544) bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) (CAS: 1689-84-5, EC: 2168827), desethylatrazine (2-amino-4-chloro-6-isopropylamino-1,3,5-triazine,) (CAS: 6190-65-4) and desisopropyla-

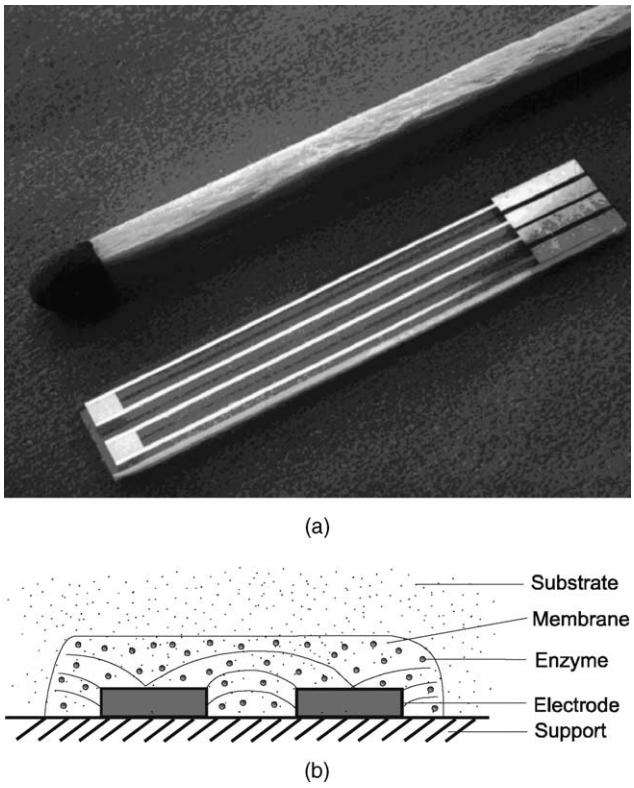

Fig. 2. (a) General view and (b) schematic representation of conductometric biosensors.

trazine (6-chloro-*N*-ethyl-1,3,5-triazine-2,4-diamine) (CAS: 1007-28-9) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All other chemicals were of analytical grade.

2.2. Sensor design

In this work, the conductometric transducers with immobilised enzymes were used to analyse the toxicity of the pollutants listed above. These sensors were fabricated at the Institute of Chemo- and Biosensorics (Munster, Germany). Two pairs of Pt (150 nm thick) interdigitated electrodes were constructed using the lift-off process on the Pyrex glass substrate (10 mm × 30 mm). A 50 nm thick intermediate Ti layer was used to improve the adhesion of Pt to the substrate. The central part of the sensor chip was closed by epoxy resin to define the electrode's working area. Both the digit width and interdigital distance were 10 μm, and their length was about 1 mm. As a result, the "sensitive" area of each electrode was about 1 mm^2 (Fig. 2).

2.3. Enzyme immobilisation

The enzymatic membrane was prepared on the transducer surface by the cross-linking of enzyme with bovine albumin in saturated glutaraldehyde vapour [25]. A mixture containing 3.5% (w/w) enzyme, 5% bovine albumin, 10% glycerol in 20 mM phosphate buffer (pH 6.0) was deposited on the sensitive area of the sensor using a drop method, while another mixture of 10% (w/w) bovine albumin and 10% (w/w) glycerol in 20 mM buffer (pH 6.0) was deposited on the other electrode. The latter electrode was considered to be the reference sensor. The sensor chips were placed in a saturated glutaraldehyde vapour for 30 min followed by drying in air for 15 min at room temperature [14]. Under the experimental conditions described above, 90% of the enzyme activity was sill obtained after 23 days of storage in a buffer solution at 4 °C.

2.4. Measurements

All the measurements were carried out in daylight and at room temperature in a vessel filled with 5 mM phosphate buffer, pH ≈ 6.0. The biosensors were dipped into the magnetic-stirred buffer solution. The differential signal was either registered by a 'home-made' conductometric laboratory amplifier for biosensor application [26] or logged by a SR 830 lock-in amplifier (Stanford Research Systems) and the steady-state response of the biosensors was plotted against the concentration of substrate.

The procedure for the evaluation of the effect of the inhibition of enzyme activity by pollutants on the biosensor response includes the following steps:

1. The biosensor was soaked in the buffer solution for 20–30 min until a stable baseline output signal was reached.
2. The 4-chlorophenol (6 mM of the final concentration) substrate was added into the measurement vessel. The corresponding steady-state output signal of the biosensor was taken as an index of the catalytic activity of the immobilised tyrosinase. This output signal value of the biosensor response was used for further evaluation of the inhibition effect of the definite concentration of pollutants.
3. After buffer washing, the sensor was incubated in the pollutant solution for 30 min.
4. After extensive washing with the buffer, the 4-chlorophenol (6 mM of the final concentration) substrate was added into the measurement vessel. Then, the inhibition rate was determined by comparing the steady-state response of the conductometric biosensors before and after exposure to a sample solution containing the toxic compounds at the substrate concentration chosen.

Each experiment was repeated at least three times.

3. Results and discussion

3.1. Substrate determination

Calibration curves obtained for two mono-phenolic substrates (phenol and 4-chlorophenol) and one di-phenolic (catechol) are shown in Fig. 3. The substrate sensitivity esti-

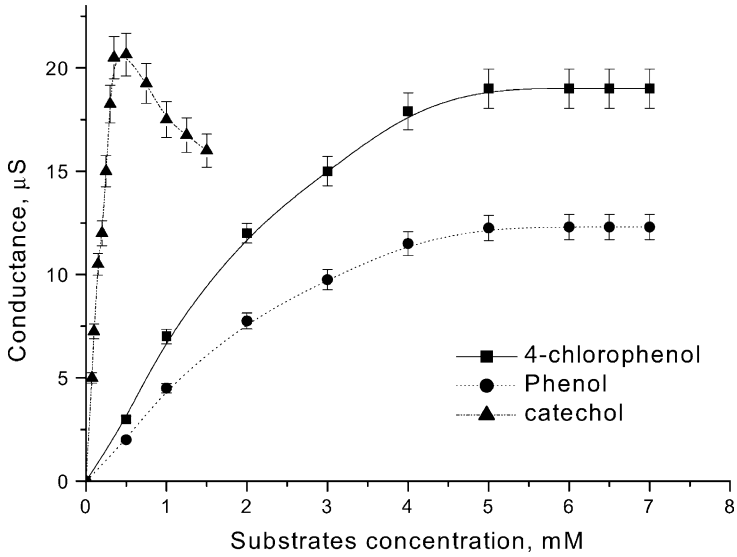

Fig. 3. Calibration curves of conductometric tyrosinase biosensor for 4-chlorophenol, phenol and catechol.

mated from the linear range decreases in the following manner: catechol > 4-chloro-phenol > phenol. Catechol could be an interesting substrate for the detection of pollutants, but for concentrations higher than 0.3 mM a decrease in the signal intensity was observed. This decrease in signal could be explained by the fact that quinones suffer from a high instability in water and they readily polymerise to polyaromatic compounds that have been shown to inactivate the enzyme and possibly foul the electrode [13,27,28]. This effect is hindered when using amperometric tyrosinase biosensor with such a substrate since quinone is permanently reduced electrochemically into catechol during the measurement. As a result the 4-cholophenol substrate was used in this work.

In order to select optimal conditions for pesticide determination, the calibration plots of the tyrosinase conductometric biosensor for 4-chlorophenol determination prior to and after incubation with atrazine were determined. As shown in Fig. 4, it is clear that the best sensitivity and accuracy of measurements were achieved using 4-chlorophenol concentrations of more than 5 mM (saturation mode for substrate). For the lower substrate concentrations, a certain amount of enzyme in the membrane was in excess and only one part of enzyme was involved in the substrate conversion to product (meaning that other parts of enzyme were not involved in the reaction). In this case the inhibited enzyme molecules could be compensated by their involvement in the reaction of another enzyme molecule. As a result, the experimental decrease of the biosensor response will be lower than the actual decrease of the enzyme activity due to the inhibition. Finally, a 6 mM concentration of the specific substrates was used in further experiments.

In this work, response time, is defined from the moment that a known volume of the substrate is injected into the measurement cell to the point at which the output signal is getting saturated. This value can vary depending on the substrate concentration used in the experiment (Fig. 5). A diffusion of the reactant (the substrate molecules) into the enzymatic membrane in which the enzymatic reaction has taken place is observed. At very low concentrations, the substrate molecules can diffuse easily into the membrane and no competition between them could occur. However, for higher concentration, there is always a small amount of substrate present in the membrane preventing the contact between the other molecules of the substrate and the catalysts (enzyme). As a result, a lengthening of the response time occurs. This is why the response time for 4-chlorophenol varies from 1 to 5 min depending on the concentration used.

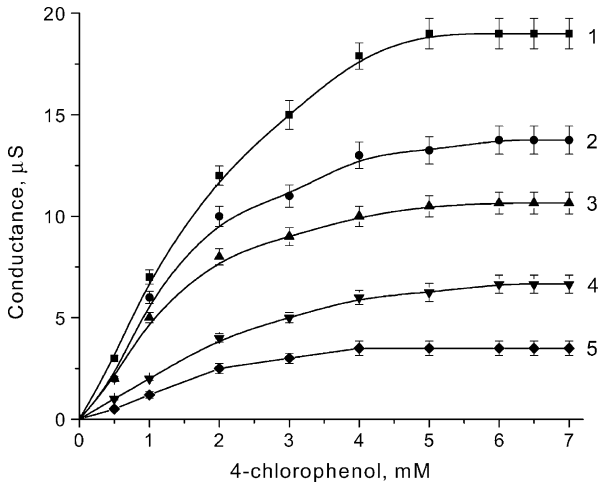

Fig. 4. Calibration curves of conductometric tyrosinase biosensor for 4-chlorophenol determination prior to (1) and after incubation with (2) 2.15 ppb, (3) 21.5 ppb, (4) 215 ppb and (5) 2.15 ppm atrazine. Measurements were conducted in 5 mM phosphate buffer, pH 6.0 with a 30 min inhibition time.

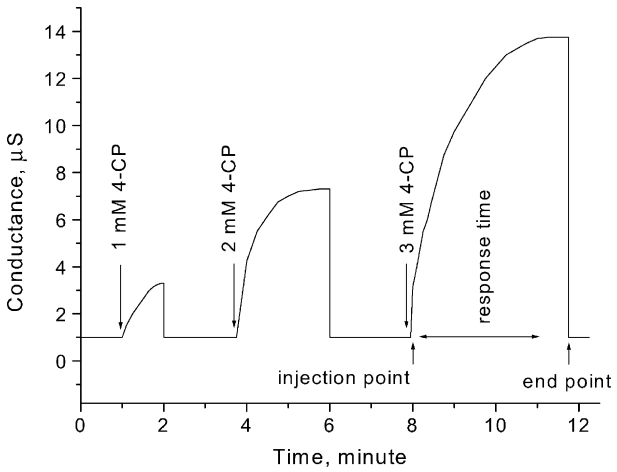

Fig. 5. Response times obtained for different concentrations (1, 2 and 3 mM) of 4-chlorophenol (temperature: 27 °C, pH 6.0).

3.2. Determination of the incubation time in pollutant solutions

The inhibition time is an another experimental parameter that influences the results and depends on which enzyme is immobilised and also on which inhibitor is detected. Evaluation of the inhibition time is very important for off-line measurements and on-site analyses. If the inhibition time is insufficient the enzymatic activity is not fully inhibited and the pollutant at very low concentrations may not be detected. In contrast, a long exposure time to the sample solution may dramatically damage the structure and properties of the enzyme. Moreover, the incubation time is a compromise between biosensor sensitivity and general time of analysis. The inhibition effect of atrazine, diuron and bromoxynil on tyrosinase is shown in Fig. 6. It can be seen in Fig. 6 that the optimal inhibition time for diuron was determined to be in the range of 20–30 min whereas for atrazine the optimum inhibition time varied from 30 to 40 min. In this work, 30 min was chosen as the contact time of the biosensor in the pollutant sample solution for both atrazine and diuron. The same experimental conditions were used on bromoxynil but no inhibition was observed on the activity of tyrosinase.

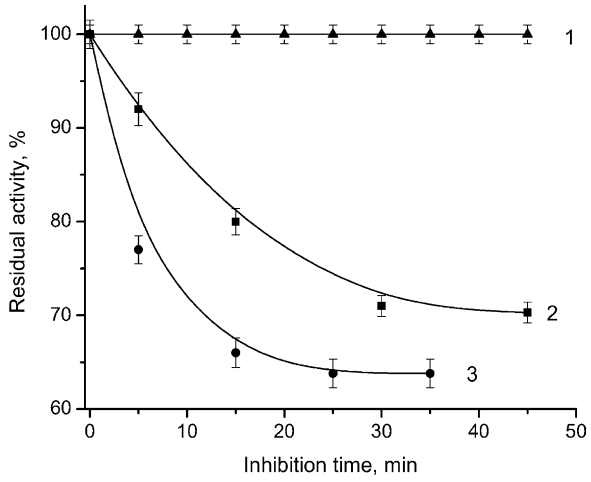

Fig. 6. Evolution of the residual tyrosinase activity versus inhibition time in solution of (1) 250 ppb bromoxynil, (2) 215 ppb atrazine and (3) 233 ppb diuron. Measurements were conducted in 5 mM phosphate buffer, pH 7.5 and 6 mM of 4-chlorophenol.

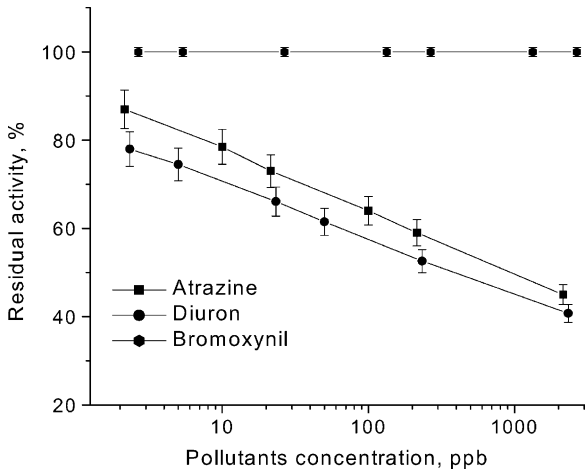

Fig. 7. Evolution of the residual tyrosinase activity vs. concentration of bromoxynil, atrazine and diuron. The biosensor responses to a 6 mM 4-chlorophenol were measured in a 5 mM phosphate buffer, pH 7.5 and an inhibition time of 30 min.

3.3. Detection of atrazine, diuron and bromoxynil

The dependence of the residual percent of tyrosinase activity versus concentration of atrazine, diuron and bromoxynil is shown in Fig. 7. It is shown that the tyrosinase activity is more inhibited for diuron than for atrazine and that in both cases a limit of detection of about 1 ppb can be reached. The limit of detection obtained in this work is far lower than the one obtained using amperometric biosensors [17,20,21]. The limit of detection was determined as the inhibitor's concentration which gives a decrease of the substrate's signal equal to three times the blank value. These results are quite encouraging since these values are close to the WHO recommendations (2 ppb) but higher than the European Community directive for herbicide detection (0.1 ppb for a single pesticide in drinking water). In contrast, bromoxynil does not affect the activity of tyrosinase meaning that this pollutant cannot be detected using this type of biosensor. The decrease of the tyrosinase activity in both cases (for diuron and atrazine) is much higher than the standard deviation (about 5%) even at a very low concentration range (2–20 ppb).

The results obtained using tyrosinase-based amperometric sensors [9,20,21] have shown that the limit of detection for phenolic substrates was 100–1000 times lower than using conductometric ones. However, the limit of detection for atrazine and diuron was 100–1000 times lower using the conductometric biosensors than the amperometric ones. This difference can be explained by considering the role

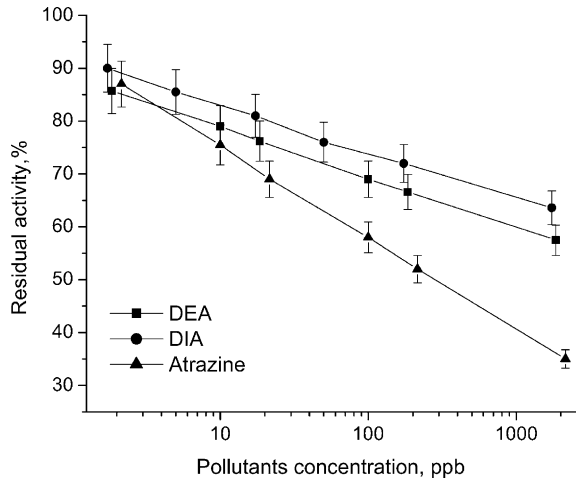

Fig. 8. Evolution of the residual tyrosinase activity vs. concentration of DIA, DEA and atrazine. The biosensor responses to a 6 mM 4-chlorophenol were measured in a 5 mM phosphate buffer, pH 7.5 and an inhibition time of 30 min.

played by the amperometric biosensors which work by reducing the generated quinone into catechol in such a way that a partial amount of substrate can be recycled, therefore, inducing an amplification on the biosensor response. As a result, this "intrinsic" amplification effect is responsible for the very low limit of detection. If inhibition takes place, the amplification does not occur and the detection of inhibitors is not as efficient. On the contrary, when using conductomeric biosensors, no amplification effect takes place and a more pronounced inhibition effect is observed.

In the environment, pesticides are usually transformed into several metabolites via different degradation processes including photo-degradation and bio-degradation among others. For instance, atrazine usually co-exists with two main metabolites called desisopropylatrazine and desethylatrazine [29] and that is why they were studied in this work. The dependence between the residual activity of tyrosinase and the concentration of atrazine, DIA and DEA is shown in Fig. 8. It can be seen in Fig. 8 that tyrosinase activity was also inhibited by the main metabolites of atrazine, DIA and DEA, but the inhibition level observed is lower than that for atrazine.

4. Conclusions

This work describes a conductometric tyrosinase-based biosensor for the detection of diuron, atrazine and its main metabolites (DEA and DIA). The limit of detection as low as 1 ppb was reached for atrazine and diuron whereas no inhibition for bromoxynil herbicide was observed.

Further, research should be continued to try and detect other phenylureas and s-triazine herbicides such as isoproturon or simazine. In addition, the interference by others substances such as the heavy ions, nitrate ions and sulphate ions should be investigated.

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