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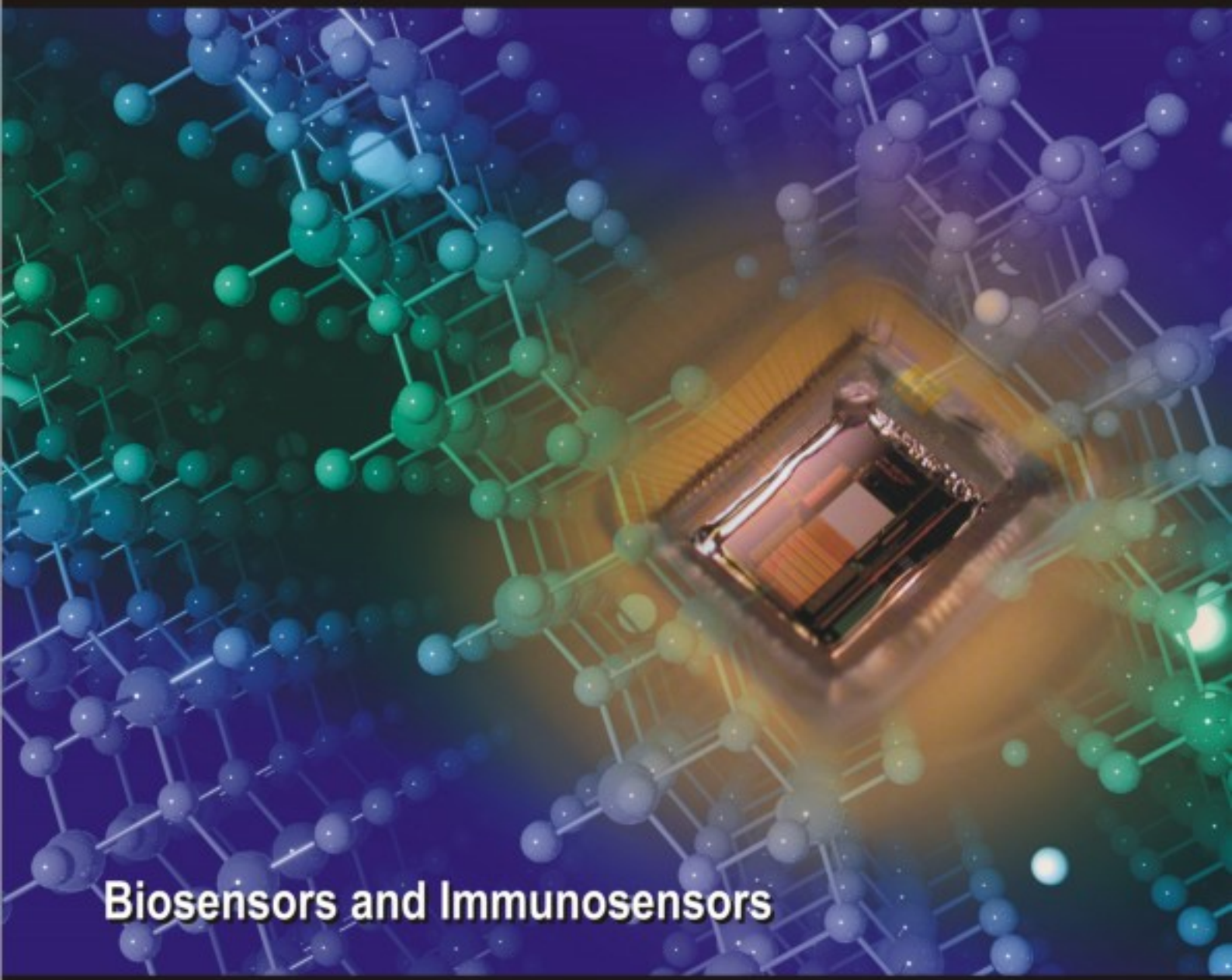

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Electrochemical Characterization of Enzymatic Impedimetric Biosensor Destined to Detect Organochlorine Pesticide: the Diclofop-Methyl

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Abstract: Preservation of aquatic ecosystems requires early warning tools such as biosensors for in situ monitoring and continuously. Such tools can provide information about the nature of pollutants condition be designed to meet specific manner. This work proposes an impedimetric biosensor, using a bacterial enzyme (Condida Rugosa lipase) immobilized in an organic matrix composed of BSA (bovine serum albumin) and glutaraldehyde, capable of detecting organochlorine pesticides (Diclofop-methyl) state trace in aqueous media. Moreover, these measurements were carried out by varying a number of parameters characteristic of the system being studied in order to better define the role of the different elements involved in the development of the receiving part of the sensor as follows: Composition of the membrane, effect of temperature and the effect of the pH of the medium. The results show that the developed biosensor provides answers in an area of very low concentrations of the order of 5.99×10^{-14} - 5.88×10^{-3} g/L and a detection limit of 5.99×10^{-14} g/L for Diclofop-methyl. This biosensor detects the pollutant in a temperature range between 20 °C and 40 °C.

We also studied the selectivity of the detection of the target substance in the presence of interfering with some heavy metals (cadmium, cobalt, zinc and lead) and other organophosphorus and organochlorines pesticide.

Keywords: Impedimetric biosensor, Condida Rugosa lipase, Organochlorine pesticide, Dichlofop-methyl, Detection.

1. Introduction

Biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological element that is in direct spatial contact with a transduction element [1]. Thus a biosensor is a combination of two elements: the bioelement and the transducer or sensor element.

Many biologically important biospecies such as enzymes, proteins and antibodies can be used as biological elements of recognition (bioelements) for biosensor [2-5]. Enzymes are large, complex macromolecules, consisting largely of protein and usually contain a prosthetic group (one or more metal atoms). Enzymes hydrolyzing triglycerides have been studied for well over 300 years, and the ability of the lipase to catalyze the hydrolysis and synthesis of esters was recognized nearly 70 years ago [6].

Lipase (triaglycerol ester hydrolase, E.C.3.1.1.3) is a member of the broad classification of hydrolases, which transfer functional groups to water. Lipases are characterized by the ability to hydrolyze the long chain triglycerides or triaglycerol (TAG) at an oil-water interface, resulting in the formation of fatty acids [7, 6].

The lipase produced by *Candida rugosa* is one of the most commonly used enzymes in organic solvent owing to its high activity in hydrolysis, esterification, transesterification and aminolysis [8-9]. Nowadays, lipase are utilized in many other applications, e.g. in the regioselective modifications of polyhydroxlic compounds, modified of oils and fats, food additives and flavours making pharmaceutical products, biodetergents, cosmetics, perfums, new biopolymeric materials, biodiesel, agrochemicals, biosensors, pesticides etc. [10]. In addition, it has been widely used in bio-transformations such as resolution of racemic acids and resolution of secondary alcohols due to the high enantioselectivity [11-13]. Due to the wide variety of environmental conditions, lipases are often easily inactivated and difficult to be separated from the reaction system for reuse. Consequently, further industrial applications of lipases are limited. By an appropriate choice of the immobilization process, operational costs for lipase industrial processes can be reduced by the selection of an appropriate immobilization method [14].

One of the most widely employed methods to pretreat an enzyme for use in organic media is to immobilize it on a solid support. Because lipases are not soluble in organic media, covalent linkages may not be necessary between the support and the lipase, and thus simple adsorption can be employed [15].

In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity, and operational stability can be modified by immobilization. Immobilized enzymes have received considerable attention because of their advantages over unimmobilized counter parts as they improve storage, operational, thermal and conformational stabilities. They can be easily recovered for reuse [16]. As the immobilization method, covalent binding, electrostatic binding, hydrophobic interactions, entrapment and encapsulation are often used for enzyme immobilization [17–21].

The immobilization of enzymes is carried out by the formation of inter- and intra-molecular cross-linkages between the enzyme molecules by means of bifunctional reagents. Glutaraldehyde has been used as a cross linker for immobilization of enzymes in which the amino groups of a protein is expected to form a Schiff base with the glutaraldehyde [22–25]. However, in terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed on supports bearing primary amino groups offers very good results in many cases, because it permits the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support.

A large number of pesticides have been used and play very important roles in agricultural production. Many of them, accounting for more than 25 % among the frequently used pesticides, are chiral compounds and consist of two or more enantiomers/stereoisomers, which may have different properties in asymmetrical environments such as living system [26].

(R,S)-2-[4-(2,4-dichlorophenoxy)] methyl propionate (diclofop-methyl) (Fig. 1) is organochlorine herbicide that present chirality; it is fatty acid synthesis inhibitor that destroy the cell membrane, prevent the translocation of assimilates to roots, reduce the chlorophyll content, inhibit photosynthesis, and have meristem activity. The diclofop-methyl (R) enantiomer shows significantly greater herbicidal activity than the (S) enantiomer [27]; therefore, to reduce the amount of herbicides used and prevent unnecessary enantiomer use causing some adverse impact, several European countries have suggested that only the active enantiomer should be employed. Consequently, there is an urgent need to develop analytical methods to determine the optical purity, stereoselective bioactivity, and environmental behavior of these chiral pesticides. Thus, several analytical methods have been used to control the enantiomeric purity of herbicides formulations, including capillary electrophoresis, immunoassays, and biosensors [28–31]. Nowadays, chromatographic and electromigration methods seem to be the most popular techniques applied in this field.

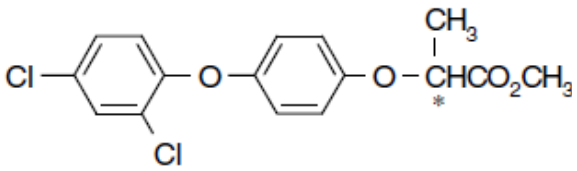


Fig. 1. Chemical structure of diclofop-methyl.

In this work, we propose to study diclofop-methyl detection by *Candida Rugosa Lipase* (CRL).

The aim of the present work was to produce an immobilized form *Candida Rugosa Lipase* with advantageous catalytic properties and stability. Lipase from *Candida Rugosa*was immobilized on the biosensor sensitive part by allowing it to mix with bovine serum albumin (BSA) and then cross-linking in saturated glutaraldehyde (GA) vapor for 30 min. The determination of pollutants in a solution was performed by comparison of the output signal of the biosensor before and after contact with pollutants, and the influence of several parameters of diclofop-methyl detection by the immobilized lipase has been studied. The measurement of the selectivity of diclofop-methyl detection was performed to study the impact of heavy metals as well as organophosphorous and organochlorine pesticides.

2. Experimental

2.1. Reagents and Solutions

All chemicals were commercially available and used as received. Candida Rugosa Lipase purchased from Sigma Chemical Co. (St. Louis, MO) was used for experiments without further purification. Bovine serum albumin (BSA), glycerol (99 %), aqueous solutions (25 % w/v) of glutaraldehyde (GA) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and organochlorine compound diclofop-methyl ((R,S)-2-[4-(2,4-dichlorophenoxy)] methyl propionate), organochlorine fungicide; chlorothalonil(tetrachloroisophthalonitrile(2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile)), herbicide compound paraquat dichloride(1,1'-dimethyl-4.4' bipyridilium dichloride), and organophosphorous compound Chlorpyriphos-ethyl(diethoxy-sulfanylidene-(3,5,6 trichloropyridin-2-yl)oxyphosphorane); all this compound were purchased from Bayer crop science and used as received, Inc. The buffer solution used for all experiments was phosphate buffered saline (PBS) containing 140 mM NaCl, 2.7 mM KCl, 0.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7,4. The reagents were of analytical grade and used as purchased without any further pretreatment. All solutions were prepared using dimeneralised water. All other chemicals were of analytical grade.

2.2. Sensor Design and Enzyme Immobilization

Platinum electrode were provided by the Laboratoire d'Analyse et d'Architecture des Systèmes (LAAS, CNRS Toulouse).The working electrode is a plate of 25 cm^2 of area.

The enzymatic membrane was prepared on the electrode surface by the crosslinking of enzyme with bovine albumin in saturated glutaraldehyde vapor [32]. A mixture containing different enzyme/support ratio [% (w/w) enzyme / % (w/w) bovine albumin], 10 % glycerol in 20 mM phosphate buffer (pH 7,4) was deposited on the sensitive area of the sensor using a drop method. The sensor chips were placed in a saturated glutaraldehyde vapor for 30 min followed by drying in air for 15 min at room temperature [33]. Biosensors were used just after preparation or stored at 4 °C in a 20 mM phosphate buffer solution, pH 7,4 until measurements.

2.3. Measurements

Impedance measurement was performed in a 30 mL three electrode electrochemical cell placed into a Faraday cage in order to improve the signal-to-noise ratio. A Platinum electrode (25 mm^2) modified with lipase (CRL) and BSA was used as a working electrode, while a saturated calomel electrode (SCE) purchased from Radiometer Analytical (Villeurbanne, France) was used as a reference electrode. The auxiliary electrode was made of a platinum wire of 1 mm diameter. Impedance measurements were performed using a Voltalab 40 instrument (Radiometer Analytical) controlled with Volta Master 4.0 software in the 100 mHz to 100 kHz frequency range, acquiring 5 points per decade. An excitation voltage of 10 mV was superimposed on a dc potential of −200 mV. Impedance data were fitted to equivalent electrical circuits by means of the ZView2 software (Scribner Associates, USA). Measurements were performed in a PBS buffer (10 Mm, pH 7,4).

Different concentrations of diclofop-methyl were injected in the PBS buffer solution to study diclofop-methyl detection by *Candida Rugosa Lipase* (CRL).

Then, the impact of heavy metals as well as organophosphorous and organochlorine pesticide was determined by comparing the steady-state response of the impedimetric biosensors before and after exposure to a sample solution containing the diclofop-methyl at the substrate concentration chosen.

All electrochemical measurements experiments were carried out at room temperature and in a faraday cage in order to eliminate electrical interferences.

3. Results and Discussion

3.1. Electrochemical Characterization of Biomembranes

First, we study the variation of the impedance spectra (the real part, it means the charge transfer resistance) of the functionalized platinum electrode with different proportion of immobilized enzyme. This is allowing us to know the response of the different enzymatic membranes immobilized on our

platinum electrode, which will leads to the high sensitivity detection. Fig. 2 shows the impedance spectra of the functionalized platinum electrode after the immobilization of enzyme membrane with different composition.

Total impedances of the bare and enzyme/BSA platinum electrodes were also determined by varying frequency in the 100 mHz to 100 kHz range. The potential was repetitively cycled until two consecutive curves could be superimposed. It took about 10 min for the bare electrode, while 20 min were needed for the modified electrode. This result confirmed that electron transfer to the electrode was slowed down by the addition of the membrane.

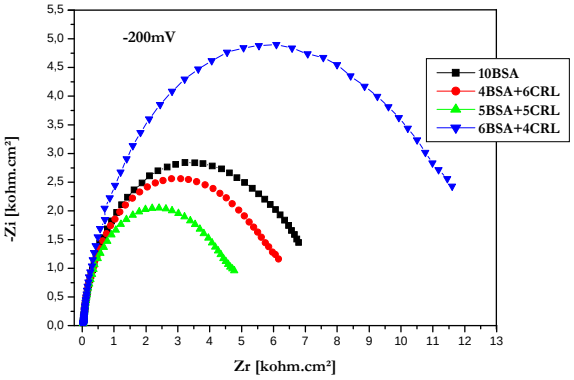


Fig. 2. Impedance spectra of the functionalized Platinum electrode after the immobilization of different compositions of enzymatic membranes in phosphate buffer saline PBS (10 mM pH7,4) at−0.2 V vs. SCE.

Fig. 3 shows the variation of the resistance versus the diclofop-methyl concentration. We can see that the surface saturation and the sensibility of the biosensor can be obtained with 4 % (w/w) Lipase (CRL) / 6 % (w/w) bovine albumin. Thus, we will use this composition. The impedance spectra can be fitted with computer simulated program using the electric circuit shown in Fig. 4b. An excellent fitting between the simulated and experimental spectra was obtained for each composition of enzymatic membranes.

Total impedances of the bare and enzyme/BSA platinum electrodes were also determined by varying frequency in the 100 mHz to 100 kHz range. The potential was repetitively cycled until two consecutive curves could be superimposed. It took about 10 min for the bare electrode, while 20 min were needed for the modified electrode. This result confirmed that electron transfer to the electrode was slowed down by the addition of the membrane.

Impedance measurements realized before and after the membrane deposition presented in the complex plan are shown in Fig. 5. It is noticed that the Nyquist diagram of a bare platinum electrode is completely different from that obtained with a modified electrode. The increase of the resistance is due to the big size of the BSA molecules (the grafted layer become more insulating). The experimental impedance spectra was recorded at -0,2 V in PBS buffer at pH=7.4 in the range of 100 mHz to 100 kHz.

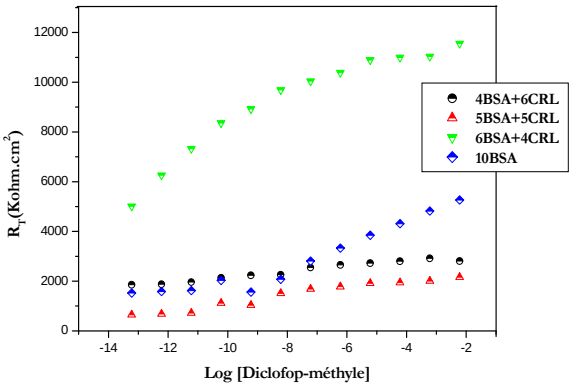
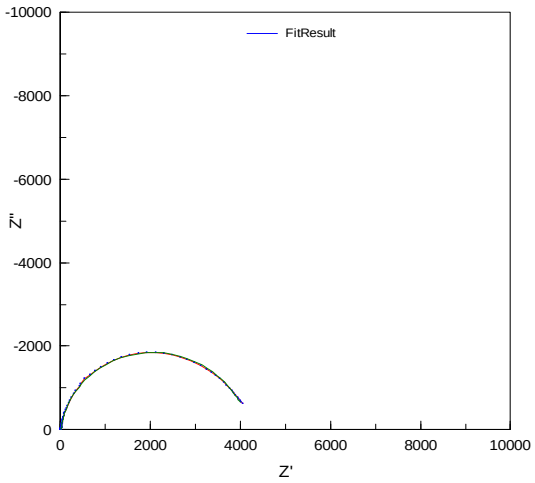
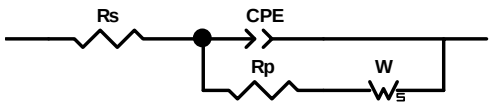


Fig. 3. Absolute value of the relative variation of the charge transfer resistance as a function of concentration of diclofop-methyl for different compositions of enzymatic membranes in phosphate buffer saline PBS (10 mM pH7,4) at−0.2 V vs. SCE.



(a)



(b)

Fig. 4. a) Impedance spectra of the Platinum/Lipase(CRL)/BSA/electrolyte interface in phosphate buffer saline PBS (10mM pH7,4) at−0.2 V vs. SCE. and in the range of 100 mHz to 100 kHz. b) Electric model.

Nyquist curve of the modified electrode could be satisfactorily fitted with The Randles–Ehrshler model, which includes Warburg impedance, Zw, resulting from ion diffusion from the bulk electrolyte

to the electrode interface, appeared as well suited (Fig. 4.b). Fitting parameters obtained for bare and modified electrodes are presented in Table 1. n values are close to 1, suggesting that CPE can be more likely considered as a capacitive element. Membrane layer significantly affected CPE and Rp. A 1,75-fold decrease of CPE and a 4,21-fold increase of Rtc were observed. Belinova et al. reported much larger variations of these parameters (2-fold decrease of CPE and 5–6 increase of Rp) for butylcholin esterase (BuChE) immobilized on gold electrodes through cross-linking with glutaraldehyde [34]. That results in a lower polarization resistance than GA-crosslinked enzymatic film.

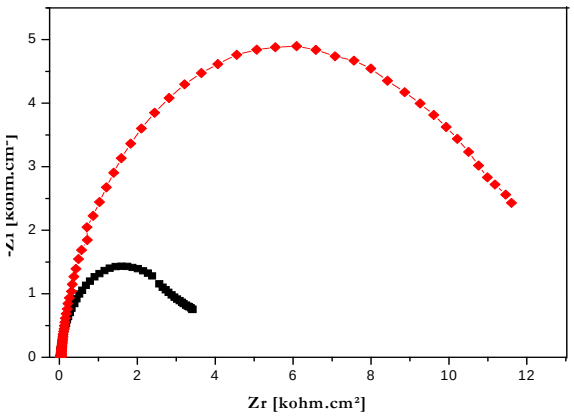


Fig. 5. Nyquist plots of the complex impedance spectra of the Platinum electrode before (■) and after (♦) CRL/BSA (4/6% (w/w)) deposition. The impedimetric measurements performed in phosphate buffer saline PBS (10mM pH7,4) at−0.2 V vs. SCE. The spectra were obtained between 100 mHz and 100 kHz. Amplitude of alternative voltages: 10 mV.

3.2. Diclofop-methyl Detection

As shown in Fig. 6, diclofop-methyl detection by lipase from Candida Rugosa immobilized on the biosensor induced a rapid decrease of resistance. The relationship between Candida Rugosa Lipase and concentration was examined in the 5.99×10^{-14}–5.88×10^{-3} g/L range. The resistance decreased linearly with diclofop-methyl concentration up to 5.90×10^{-5} g/L and a progressive saturation was observed beyond this value (Fig. 7). This linear domain is much wider than that of other composition of biomembrane. Limit of detection was 5.99×10^{-14} g/L.

3.3. pH Effect on Biosensor Response

The pH value is an important parameter for this study. Fig. 8 an increase of the capacitance of the Pt/membrane is observed when pH value increases until 8, beyond this value, the capacitance decreases. Under alkaline conditions, diclofop-methyl rapidly hydrolyzes into diclofop-acid, which has higher solubility in water and lower acute toxicity than its parent compound.

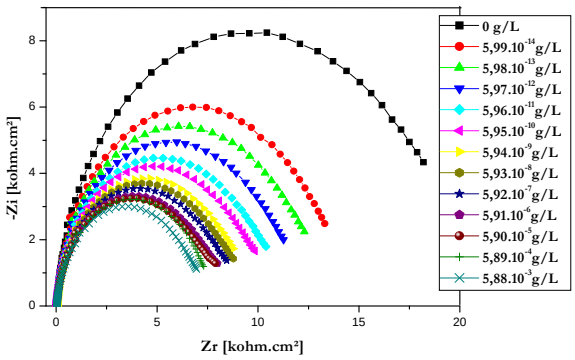


Fig. 6. Nyquist diagrams of the functionalized platinum electrode after addition of different concentration of diclofop-methyl. Measurement medium, frequency range, ac voltage, dc applied potential,-0.2V/SCE.

3.4. Temperature Influence on the Response Biosensor

Fig. 9 shows the variation of response of biosensor as a function of solution temperature. The response for diclofop-methyl showed an optimum at 30°C. Thus, it is clear that this will be chosen for further measurement. At this temperature, the biosensor is sensitive.

3.5. Selectivity of the Biosensor

The biosensor response was examined in the presence various interfering elements. Figs. 10-16 present the response of the biosensor for different interfering elements. The highest interfering sensitivity was found on lead, followed by Zinc, Paraquat and Chlorothalonil, than the Cobalt whereas there is no interference for Cadmium and Chlorpyriphos-ethyl.

Table 1. Values of equivalent circuit elements obtained by modeling the Nyquist curves presented in Fig. 5. The best fit was achieved by minimizing the χ^2 (khi square) parameter using the ZView2 software.

Electrode	R_S (kΩ)	CPE-T $\mu F(rad/s)^{l-n}$	n	R_{tc}(kΩ)	W_0-R	W_0-T	W_0-P	χ^2 ($\times10^{-3}$)
Bare platinum electrode	20.32	41.2	0.93	3.136	73025	6518	0.57	2.8
4BSA+6CRL/Pt	33.34	23.6	0.91	13.202	117.8	4.716	0.99	3

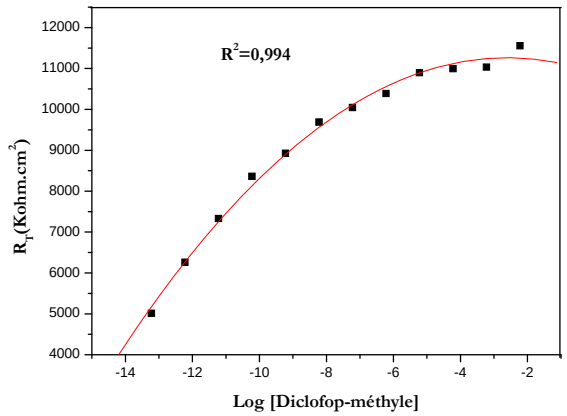


Fig. 7. Calibration curves describing the variation of resistance Rtc against the diclofop-methyl concentrations.

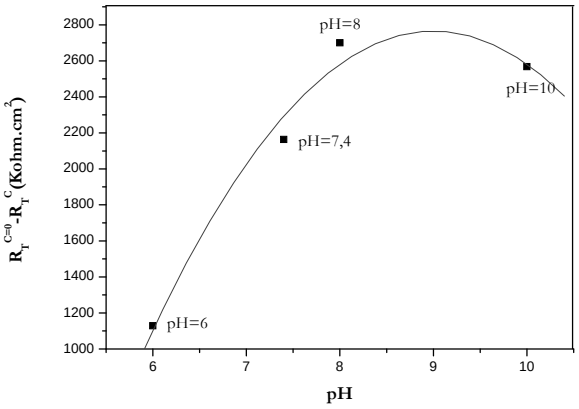


Fig. 8. pH influence on the response of the biosensor in a 5.89×10^{-4}g/L solution.

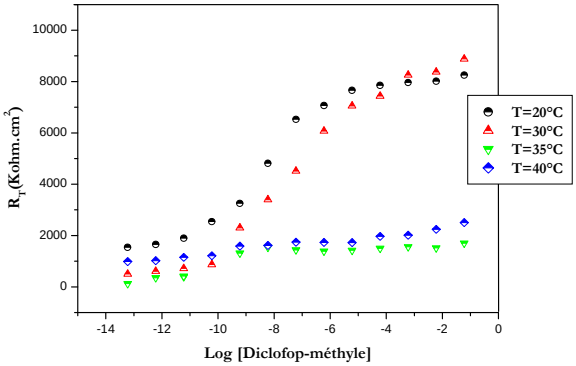


Fig. 9. Temperature influence on the response of the biosensor.

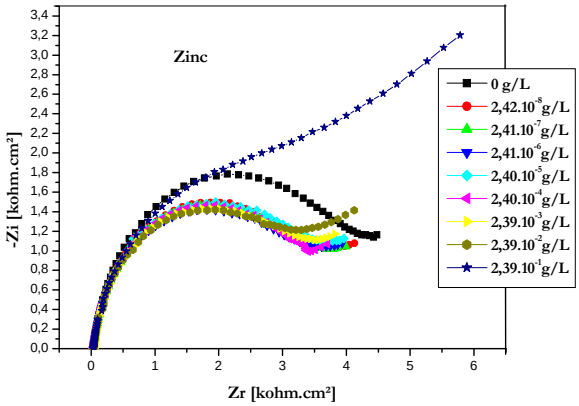


Fig. 10. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Zinc.

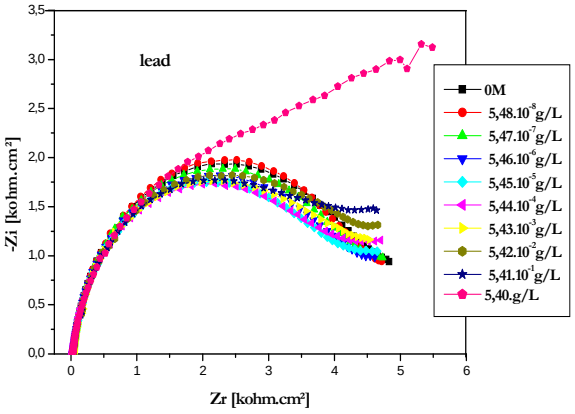


Fig. 11. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Lead.

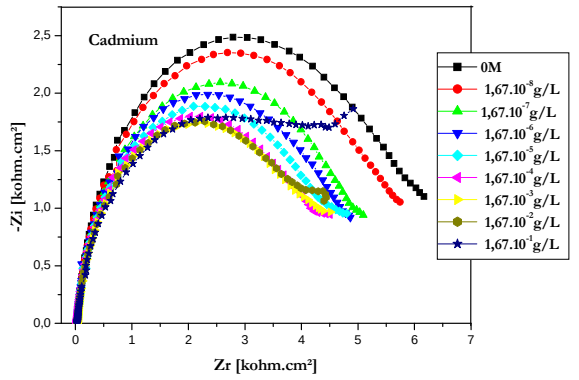


Fig. 12. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Cadmium.

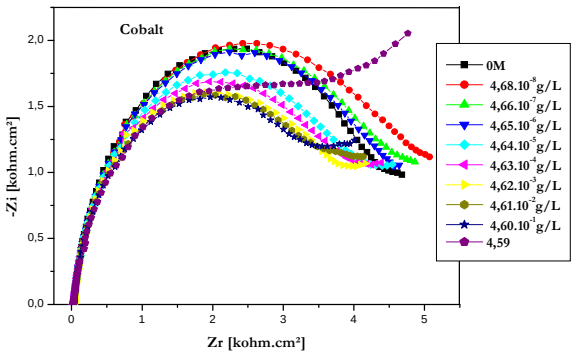


Fig. 13. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Cobalt.

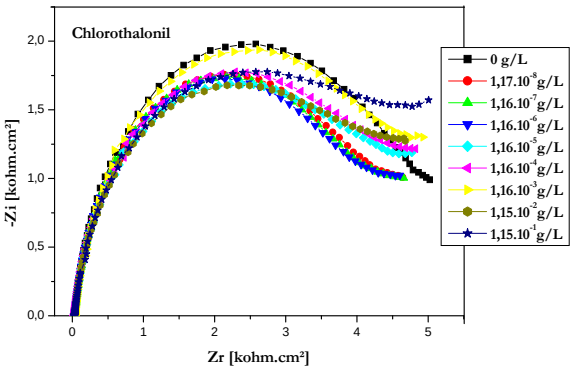


Fig. 14. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Chlorothalonil.

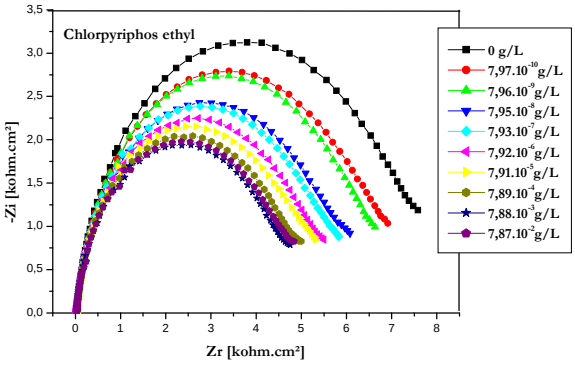


Fig. 15. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Chlorpyriphos-ethyl.

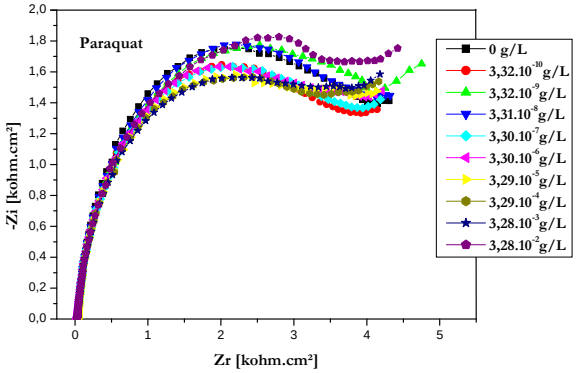


Fig. 16. Nyquist diagrams of the functionalized platinum electrode after addition of different concentrations of interfering element Paraquat.

4. Conclusion

This work describes an impedimetric lipase-based biosensor for the detection of diclofop-methyl. The main analytical characteristics of the biosensor created depend on conditions of membrane composition, pH and temperature of solution. The limit of detection as low as 5.99×10^{-14}g/L was determined for diclofop-methyl. The biosensor was tested for organophosphorous and organochlorine pesticide either some heavy metals detection.

The results presented in this work demonstrate for the first time that Candida Rugosa lipase biosensors for determination of diclofop-methyl are feasible.

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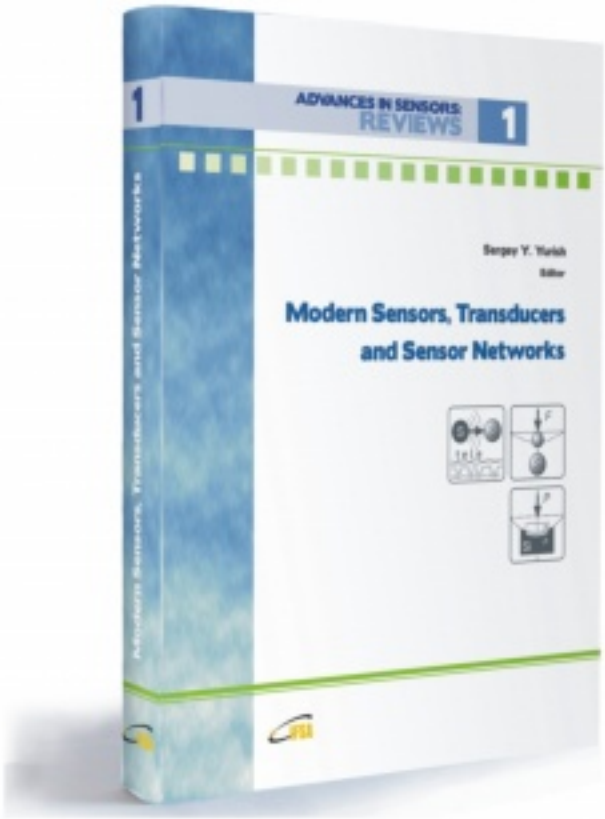

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