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Analytical Methods

Ultra-sensitive biosensor based on genetically engineered acetylcholinesterase immobilized in poly (vinyl alcohol)/Fe–Ni alloy nanocomposite for phosmet detection in olive oil

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ABSTRACT

An ultra-sensitive screen-printed biosensor was successfully developed for phosmet detection in olive oil, based on a genetically-engineered acetylcholinesterase (AChE) immobilized in a azide-unit water-pendant polyvinyl alcohol (PVA-AWP)/Fe–Ni alloy nanocomposite. Fe–Ni not only allowed amplifying the response current but also lowering the applied potential from 80 mV to 30 mV vs Ag/AgCl. The biosensor showed a very good analytical performance for phosmet detection, with a detection limit of 0.1 nM. This detection limit is lower than the allowable concentrations set by international regulations. In addition to the good reproducibility, operational and storage stability, the developed biosensor was successfully used for the determination of phosmet in olive oil samples without any laborious pre-treatment. The phosmet recovery rate was about 96% after a simple liquid–liquid extraction.

1. Introduction

Organophosphate (OP) compounds are widely used as insecticides for crop protection and as chemical warfare agents. They represent more than 38% of the total pesticides used worldwide (Singh, 2009). Commonly used OPs include parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos and azinphos methyl. Phosmet (2-(dimethoxyphosphinothioylsulfanylmethyl)isoindole-1,3-dione) (Fig. 1) is a very efficient organophosphorus insecticide which has been commonly used around the world for controlling a number of insects on horticultural crops and plants (Belmonte Valles, Retamal, Mezcua, & Fernández-Alba, 2012; Hernández-Borges, Cabrera, Rodríguez-Delgado, Hernández-Suárez, & Saúco, 2009). One of these insects is olive fruit fly (*Bactrocera oleae* Gml.), which attacks olive trees and causes significant quantitative and qualitative losses in olive oil production. Taking into consideration that olive oil is a high added value product, the protection of olive trees appears as a priority (Cunha, Fernandes, Beatriz, & Oliveira, 2007). On the other hand, OP residues that remain in the oil and fruits are considered a major risk for consumer health. As a consequence, the European Union (Regulation EC No 396/2005) and the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO) recommend a maximum residue limit (MRL) of 3 mg/kg in olives products (Codex Alimentarius Commission, 1996).

Developing simple, rapid and accurate methods has become urgent to trace these toxic substances in various media like air, water and food. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) are common conventional methods employed for pesticides determination that have been reported in regulations to monitor the environmental pollutants (Van der Hoff & van Zoonen, 1999 and Mitobe, Ibaraki, Tanabe, Kawata, & Yasuhara, 2001). Although these methods provide accurate results, these are cumbersome and time-consuming, particularly in sample preparation. Furthermore, these approaches are reserved to highly specialized laboratories having very expensive equipment and trained personnel. This fact encouraged scientists to find out other techniques overcoming these drawbacks, among which biosensors appear as one of the most promising approaches, providing many advantages such as simplicity, rapidity, low analysis cost, relatively economic equipment, and user-friendly operation.

Many enzyme-based electrochemical sensors have been already described for the detection of pesticides belonging to carbamate

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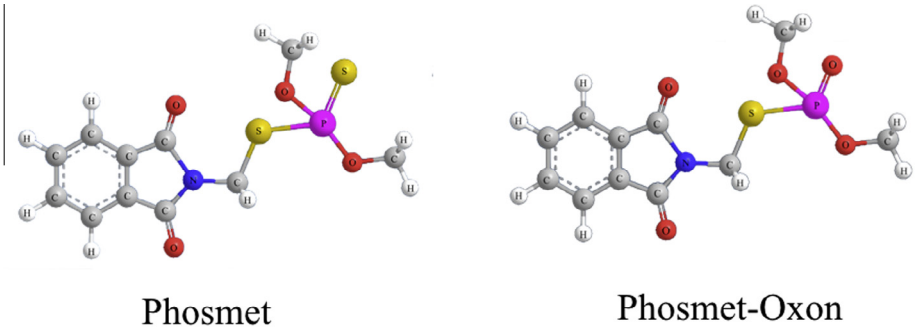


Fig. 1. Chemical structures of phosmet and phosmet-oxon.

and organophosphorus classes. The concept of these biosensors is generally based on the ability of these pesticides to inhibit acetylcholinesterase (AChE) reaction (Cesarino, Moraes, Lanza, & Machado, 2012; Pohanka, Musilek, & Kuca, 2009; Wang, Gu, Zhang, Zhang, & Zhu, 2009). However, despite intense progress has been achieved in electrochemical sensor research, a few AChE sensors have been applied to the detection of OP pesticides in real samples due to their lack in sensitivity, stability or selectivity. In recent years, the development of such biosensors has been based on two strategies, acting either on the biological element or the transducer. The first consists of using genetically-modified enzymes that have been optimized for increasing the biosensor selectivity and sensitivity to inhibitors (De Oliveira Marques, Nunes, Dos Santos, Andreescu, & Marty, 2004; Nunes, Montesinos, Marques, Fournier, & Marty, 2001; Sotiropoulou, Fournier, & Chaniotakis, 2005). The second strategy involves transducer modification by chemicals such as electrochemical mediators, for improving the quality and selectivity of output signal (Istamboulié et al., 2010). In the recent years, a special attention has been paid to nanomaterials, especially in form of nanoparticles or nanotubes, due to their unique structural and functional properties such as high surface area and high electrical conductivity, that enhance the electron transfer between the enzyme redox center and the electrode surface (Cesarino et al., 2012). For instance, a biosensor for paraoxon was developed based on the use of gold nanoparticles, allowing a negative shift of applied potential and a great signal amplification, leading respectively to a higher selectivity and sensitivity (Wang et al., 2011). Similarly, Du and coworkers developed an AChE-based biosensor incorporating CdTe quantum dots and gold nanoparticles; they reported that this modification allowed a dramatic improvement of sensitivity, the limit of detection (LOD) for monocrotophos insecticide being 1.34 μM (Du, Chen, Song, Li, & Chen, 2008).

This study describes the development of a biosensor based on genetically-modified acetylcholinesterase immobilized in photocrosslinkable poly (vinyl alcohol) and modified with Fe-Ni alloy nanopowder. The obtained biosensor was applied to the detection of phosmet pesticide after rapid extraction from olive oil.

2. Materials and methods

2.1. Chemicals, enzymes and solutions

Both acetylcholinesterases from *Drosophila melanogaster*: wild type enzyme (B131) and genetically modified enzyme (B394) were produced by the Centre de Recherche de Biochimie Macromoléculaire (Montpellier, France) (Boublik et al., 2002). Acetylcholinesterase (EC 3.1.1.7) from electric eel (EE) (Type V-S, 1,000 U/mg), iron-nickel alloy, 55:45 nanopowder <100 nm (PET) (Fe–Ni NP), acetylthiocholine chloride (ATChCl), and all other chemicals were purchased from Sigma Chemical Co. (Germany). All solutions used in the experiments were prepared daily and stored at 4 °C. Photocrosslinkable poly (vinyl alcohol) (azide unit pendant water-soluble photopolymer, PVA-AWP) was purchased from Toyo Gosei (Japan). Phosmet-oxon solution in iso-octane was from Dr. Ehrenstorfer (Augsburg, Germany). Graphite (Electrodag 423SS) and silver/silver chloride (Electrodag 418SS) inks were obtained from Acheson (Plymouth, UK). Cobalt phtalocyanine-modified carbon paste was purchased from Gwent Electronic Materials, Ltd. (Gwent, UK). Poly (vinyl) chloride (PVC) sheets (200 mm × 100 mm x 0.5 mm), supplied by SKK (Denzlingen, Germany), were used as support for the screen-printed electrodes. A glycerophtalic paint (Astral, France) was used as insulating layer.

2.2. Determination of acetylcholinesterase activity

The studies of AChEs activity were carried out with a SHIMADZU UV-1800 spectrophotometer. Enzyme kinetics were measured using Ellman's method (Ellman, Courtney, Andres, & Featherstone, 1961), which is based on the reaction between the reaction product thiocholine and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), leading to a yellow compound (5-thio-2-nitrobenzoate) absorbing at 412 nm (ε = 13,600 M^{-1} cm^{-1}). 1 enzymatic unit (U) was defined as the amount of enzyme hydrolysing 1.0 μmole of acetylthiocholine per min.

2.3. Determination of the inhibition constant (k_i)

The inhibition mechanism of organophosphate compounds on AChE is well-known (Aldridge, 1950). The OP binds covalently to a serine residue, leading to an irreversible inhibition of the enzyme:

$$\underset{\text{(Enzyme)}}{\text{E-OH}} + \underset{\text{(OP)}}{\text{P-X}} \rightarrow \underset{\text{(Phosphorylated enzyme)}}{\text{E-OP}} + \text{X-OH (X = leaving group)}$$

Such inhibition can be followed by varying the incubation time of enzyme and inhibitor. The inactivation of enzyme follows a pseudo-first order kinetic (Worek, Thiermann, & Szinicz, 2004; Worek, Thiermann, Szinicz, & Eyer, 2004):

$$v = -d[\text{E}]/\text{dt} = -d[\text{OP}]/\text{dt} = k_i[\text{E}][\text{OP}]$$

The procedure used for the determination of inhibition constants (k_i) was already described (Villatte, Marcel, & Estrada-Mondaca, 1998), it was adapted from the methodology described by Segel (1975). In this work, the inhibition constants k_i were determined for the three types of AChE as follows: AChE was incubated for 0, 10, 30, 50, 70 and 90 s with different concentrations of pesticides, then the enzyme activity was determined spectropho-

tometrically as described above. The remaining activity of AChE was calculated by comparing the obtained kinetic slope before and after inhibition. The graph obtained by plotting log of residual activity versus incubation time shows a linear representation, whose slope allows calculating the apparent reaction rate k_{obs} (min^{-1}). Plotting $1/k_{obs}$ versus 1/[OP] allows calculating the inhibition constant k_i for each enzyme, which corresponds to the reciprocal value of the obtained slope.

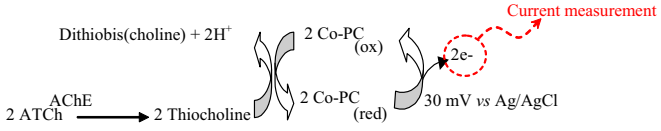
2.4. Biosensor construction

Screen-printed electrodes were fabricated using a semi-automatic DEK248 printing machine according to a procedure previously described (Andreescu, Barthelmebs, & Marty, 2002), but in a three-electrode configuration. The working electrode incorporating cobalt phtalocyanine as mediator was a 4 mm-diameter disk, the auxiliary electrode was a 16 mm × 1.5 mm curved line and the Ag/AgCl pseudo-reference electrode was a 5 mm × 1.5 mm straight track. The enzyme was immobilized into a poly(vinyl alcohol)-based photopolymer (AWP) by physical entrapment as reported previously (Andreescu et al., 2002). A homogenous mixture of AChE B394 and PVA-AWP was prepared in a ratio 30:70 in presence of different amounts of Fe–Ni NP, and 3 μL of this mixture were deposited on the surface of the modified working electrode. The amount of AChE enzyme immobilized on the working electrode was 1 mU. The electrode was kept under neon light for 3 h at +4 °C to allow complete crosslinking.

2.5. Electrochemical measurements

The behavior of each type of sensor was studied by cyclic voltammetry in a potential range from −200 mV to 500 mV at a scan rate of 10 mV/s in 0.1 M phosphate buffer pH 7 (KH_2PO_4/Na_2HPO_4 + KCl 0.1 M) containing 1 mM acetylthiocholine chloride (ATChCl). These studies allowed studying the electrochemical effect of Fe–Ni NP as well as determining the working potential.

The reactions involved in an AChE biosensor are described in the following scheme:



The flow of electrons is proportional to the rate of acetylthiocholine hydrolysis, which decreases upon phosphorylation of a serine present in the enzyme active site by OP. Amperometric measurements were carried out with a 641 VA potentiostat (Metrohm, Switzerland). The current was measured with a BD40 (Kipp & Zonen, The Netherlands) flatbed recorder. The screen-printed electrode system was immersed in a cell containing 5 mL phosphate buffer pH 7 under constant magnetic stirring; the applied potential was 30 mV vs Ag/AgCl reference electrode. After current stabilization, the current intensity was recorded and 1 mM ATChCl (final concentration) was added in the cell. The measured signal corresponded to the difference of current intensity between the baseline and the plateau. The pesticide detection was performed by measuring the residual response of the biosensor after a 10-min incubation in a solution containing a known concentration of insecticide.

Both electrodes and cell were thoroughly washed between assays with distilled water. The inhibition percentage was related with the insecticide concentration, it was calculated according to the following formula:

$$I(\%) = [(A_0 - A)/A_0] \times 100$$

Where A and A_0 are the currents measured respectively after and before inhibition.

2.6. Extraction of pesticides from olive oil

Before assays, the pesticides were extracted using a simple liquid–liquid extraction procedure as reported by Ben Oujji et al. (2013) slightly modified as follows: 10 mL of a commercially available organic extra virgin olive oil purchased in a local supermarket was spiked with a known concentration of pesticide diluted in isooctane. The mixture was heated at 50 °C for 30 min, and 100 μL of this olive oil was mixed with 900 μL of 3 types of extraction solvents: Acetonitrile, acetonitrile:dichloromethane (8:1, v:v), acetonitrile:ethyl acetate (8:1, v:v). The mixture was centrifuged at 12,000*g* for 90 s using an Eppendorf Minispin centrifuge, the resulting supernatant was recovered and used as a pesticide mother solution.

This study focuses on the detection of phosmet-oxon, which is the oxidized form of phosmet, it is less stable but much more toxic than the parental form. The oxidation step was carried out using N-bromosuccinimide (NBS) (Kralj, Trebše, & Franko, 2006). It was shown that a 10^{-6} M NBS solution was efficient for the total oxidation of phosmet solution. The effect of NBS on the enzyme activity was also tested, it was found that NBS did not have any denaturing or inhibiting effect on AChE in the used conditions.

3. Results and discussion

3.1. Determination of inhibition constants (k_i)

The inhibition constant k_i is an indicator of the pesticide affinity for the enzyme. This parameter reflects the inhibitory power and is thus used to compare the potency of insecticides and to assess the sensitivity of the enzymes studied. Most of the biosensors described in literature are based on the classical acetylcholinesterase from electric eel (*Electrophorus electricus*); other studies involve human or horse acetyl- and butyryl- cholinesterases, but the resulting sensors showed low sensitivities to organophosphorus insecticides (Galezowska et al., 2008). In this work, the sensitivity of electric eel AChE was compared to drosophila wild type and mutant acetylcholinesterases.

As shown in Table 1, AChE B394 mutant enzyme displayed the highest sensitivity to phosmet-oxon, its inhibition constant k_i is 28 and 379-fold higher than those of wild type drosophila and electric eel enzymes, respectively. Based on these results, the present work was focused on the development of enzyme sensors based on the genetically-modified acetylcholinesterase B394.

3.2. Effect of Fe–Ni NP and optimization of applied potential

Different biosensors containing or not Fe–Ni NP were fabricated. As shown in Fig. 2, cyclic voltammetry experiments show that the presence of Fe–Ni NP allow dramatically enhancing the

Table 1
Inhibition constants k_i calculated for the three acetylcholinesterases.

Acetylcholinesterase	k_i ($\mu mol^{-1}\ min^{-1}$)
Electric eel (wild type)	1.1
Drosophila (wild type)	15.5
Drosophila (B394 mutant)	416.7

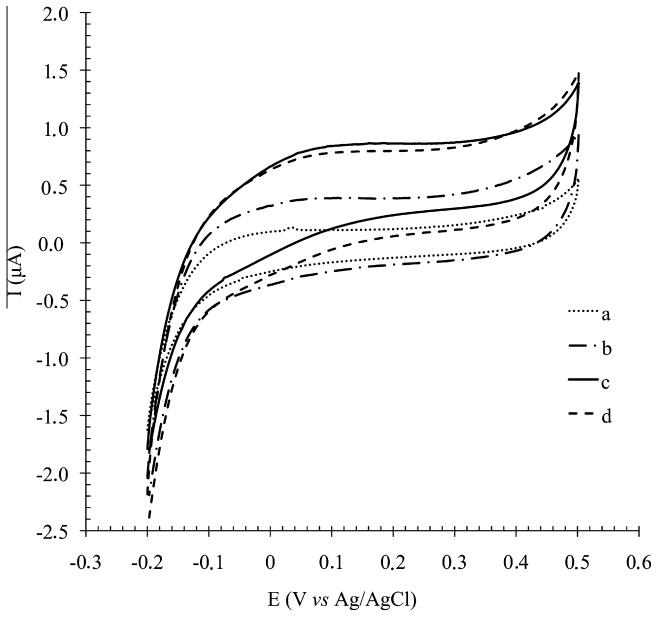


Fig. 2. Cyclic voltammograms: (a) PVA-AWP/Fe–Ni NP/AChE B394 in 0.1 M phosphate buffer pH 7; (b) PVA-AWP/AChE B394 in 0.1 M phosphate buffer pH 7; (c) PVA-AWP/AChE B394 + 1 mM ATChCl; (d) PVA-AWP/Fe–Ni NP/AChE B394 +1 mM ATChCl.

electrochemical response of the biosensor and led to amplification of the current response. The current response of the biosensor incorporating 10 µg of nanoparticles per electrode was shown to be two fold greater than the response of the classical biosensor. This enhancement was not only due to the excellent conductivity characteristic of Fe–Ni NP (high surface free energy and high electron transfer), but also to the large surface area of Fe–Ni NP which contributes to increase the enzyme surface loading (Fangqiong, Xianwei, Dong, Junguo, & Changqiong, 2000; Ou et al., 2007; Zhao, Zhang, Bai, Yang, & Sun, 2006). These results are in good agreement with previous works reporting the use of copper, nickel, or gold nanoparticles to enhance the sensitivity of biosensors (Heli, Hajjizadeh, Jabbari, & Moosavi-Movahedi, 2009; You et al., 2003; Zhao et al., 2015).

One of the most important factors affecting the selectivity and sensitivity of electrochemical biosensors is the applied potential. The biosensor response to 1 mM ATCh was recorded at applied potentials ranging from -210 to 100 mV vs Ag/AgCl in 0.1 M PBS. As shown in Fig. 3, a continuous increase of the PVA-AWP/AChE biosensor response was observed from −210 to 80 mV, where a maximum value was reached at 80 mV. When Fe–Ni NP were

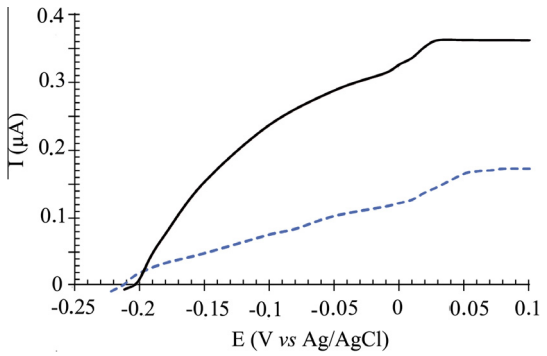


Fig. 3. Effect of Fe–Ni NP on the applied potential: PVA-AWP/Fe–Ni NP/AChE B394 (solid line) and PVA-AWP/AChE B394 (dotted line).

added in the electrode (10 µg NP/electrode), this maximum response was achieved at 30 mV vs Ag/AgCl. Such negative shift in applied potential allows enhancing the selectivity of the biosensor by minimizing the risk of interference due to oxidation of other electroactive species, which are likely to be present in real samples. Furthermore, this low-oxidative applied potential allows lowering background current and noise levels to their minimum values (Wei, Liu, Li, & Chen, 2011).

3.3. Analytical performance of the biosensor

Experiments were first carried out to verify the reproducibility between the amperometric responses provided by 5 biosensors prepared in the same manner. It was shown that the inter-variability between electrodes was lower than 5%, whatever the type of electrode used. Under the optimal conditions, calibration curves were drawn using phosmet-oxon concentrations varying from $8 \cdot 10^{-11}$ M ($2.4 \cdot 10^{-5}$ ppm) to $5 \cdot 10^{-9}$ M ($1.5 \cdot 10^{-3}$ ppm). The inhibition ratio was determined for each concentration after a 10 min incubation of the biosensor in the pesticide solution. The obtained results showed a very good analytical performance. As can be seen in Fig. 4, the semi-log linear representation shows a linear response in the studied concentration range with a correlation coefficient $r^2 = 0.992$ ($n = 8$). The biosensor stability was tested by performing 15 consecutive measurements, an excellent reproducibility was observed, with a mean response current of 376 ± 5 nA. Storage stability was also tested within the period of experiments, and the biosensor activity was shown to be stable for more than 1 month of storage at +4 °C in sealed plastic boxes.

The developed biosensor showed a LOD of 0.1 nM, corresponding to $3 \cdot 10^{-5}$ ppm, which is lower that those achieved using similar biosensors involving other mediators like poly(3,4-ethylenedioxythiophene) (PEDOT) (LOD = 4 nM chlorpyrifos-oxon) (Istamboulie et al., 2010) or tetracyanoquinodimethane (LOD = 2.3 nM chlorpyrifos-oxon) (Andreescu et al., 2002). The achieved LOD is highly compatible with international regulations, wich establish the MRL for phosmet in olive oil at 3 ppm.

3.4. Olive oil analysis

The developed biosensor incorporating Fe–Ni NP was tested to detect insecticide extracted from spiked olive oil. Assays were performed in triplicate. The olive oil samples were firstly analyzed before pesticide spiking, confirming the absence of other insecticide and any other potential interfering component of oil. The samples were then spiked with phosmet and phosmet-oxon at concentrations of 10^{-7} M, $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-8}$ M. After liquid–liquid extraction, 100 µL of the extracted solution were injected in the cell. The results obtained for phosmet-oxon detection showed a maximum recovery rate using acetonitrile for liquid/liquid extraction. This solvent was thus used for phosmet extraction, and 100 µL of the resulting extract were injected in the cell in presence of 10^{-6} M NBS as oxidizing agent. Surprisingly, no inhibition was observed whatever the concentration of the doping solution. The same experiment was carried out using 100 µL of 10^{-5} M phosmet solution instead of spiked olive oil. The pesticide was extracted using 900 µL acetonitrile or (acetonitrile:dichloromethane 8:1), and 100 µL of extract was injected in the cell in presence of 10^{-6} M NBS. No inhibition was observed whatever the solvent used, while HPLC analysis demonstrated that phosmet was properly extracted (96%). It was therefore concluded that phosmet extraction was effective but its oxidation by NBS was not possible in these conditions. Other extraction solvents were thus investigated, namely acetonitrile:acetone, 8:1 (v:v) and acetonitrile:ethyl acetate, 8:1 (v:v). In these cases, inhibition effects were observed, showing that the efficiency of NBS oxidation was

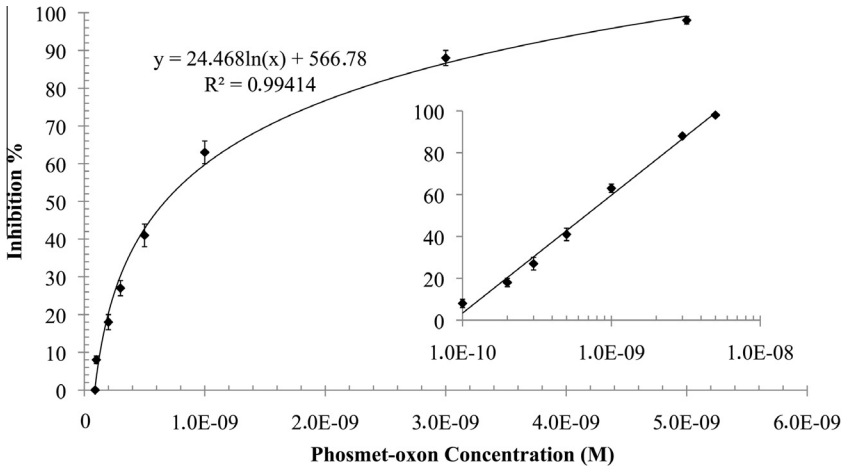


Fig. 4. Calibration curve obtained for the amperometric detection of phosmet-oxon using PVA-AWP/Fe-Ni NP/AChE B394 biosensor (window: semi-log plot).

solvent-dependent. These two solvent mixtures were thus applied to extraction of phosmet from spiked olive oil. After biosensor analysis, the obtained recovery yields were calculated to be respectively 47% and 96% for acetonitrile:acetone and acetonitrile:ethyl acetate. These results demonstrate that the PVA-AWP/Fe–Ni NP/AChE B394 biosensor can be efficiently used for the detection of phosmet and phosmet-oxon in olive oil samples after a simple liquid–liquid extraction.

4. Conclusion

In this work, a fast and simple amperometric biosensor was constructed using a genetically modified acetylcholinesterase, highly sensitive to phosmet, which was immobilized in a PVA-AWP/Fe-Ni nanopowder. The use of Fe–Ni NP allowed not only working at low potential, thus avoiding interferences, but also amplifying the response current by more than two folds when compared to non-modified biosensor. The developed biosensor allowed detecting phosmet at concentrations ranging between $1 \cdot 10^{-10}$ M and $5 \cdot 10^{-9}$ M. Olive oil samples were analyzed using the biosensor after a simple liquid–liquid extraction from olive oil, and the achieved results were in good agreement with those obtained by HPLC analysis.

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