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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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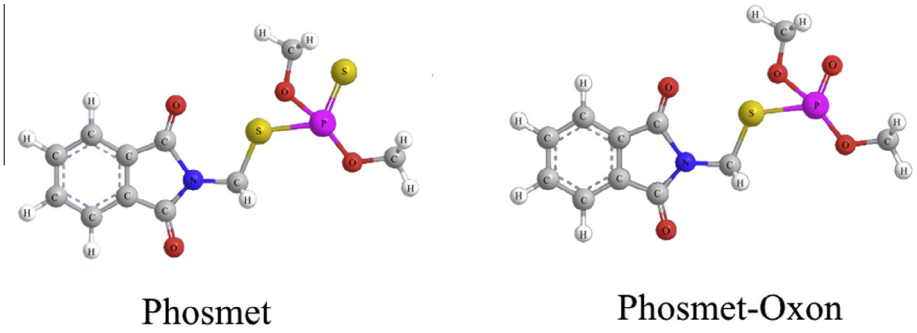

Fig. 1. Chemical structures of phosmet and phosmet-oxon.

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-